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The nucleotide sequence preceding and including the beginning of the ilvE gene of the ilvGEDA operon of Escherichia coli K12

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ABSTRACT

The DNA sequence of the 570 base pairs that precedes and includes the beginning of the ilvE gene shows no evidence of a leader-attenuator region. Instead, the sequence shows that the ilvE gene is preceded by another structural gene, presumably the normally cryptic ilvG gene. In vitro transcription of two plasmids (pLC26-3 and pRL5) containing the regulatory region of the ilvEDA operon results in the formation of two "leader" RNAs. These results are consistent with the suggestion that the ilvEDA operon is regulated by an attenuator mechanism and that the attenuator region lies before the ilvG gene.

INTRODUCTION

The organization of the genes for the biosynthesis of isoleucine and valine has recently undergone extensive revision and clarification. Five of these genes were mapped initially as a cluster, ilvBCADE, (18) at a position corresponding to 83 min on the current map of the Escherichia coli K12 chromosome (2). These genes are expressed as three separate units of transcription, ilvB, ilvC, and ilvADE (24). The ilvB gene specifies the enzyme acetohydroxy acid synthase I (24) (Fig. 1). Another isozyme, acetohydroxy acid synthase III, specified by the ilvHI gene is located in a region linked to the leu operon (24). Both of these isozymes, which are normally expressed in E. coli K12, are subject to feedback inhibition by valine (8). Consequently, growth of this strain is inhibited by valine due to isoleucine starvation.

E. coli K12 can become resistant to the growth-inhibiting effects of valine by several mechanisms (10). One mechanism originally observed by Ramakrishnan and Adelberg is via an ilvO mutation (18). These cis dominant mutations result in derepressed levels of the enzyme activities specified by the ilvE, D, and A genes and a simultaneous resistance to the growth-inhibiting effects of valine. Ramkrishnan and Adelberg (18) mapped these

mutations between ilvA and ilvC. These data led to the conclusion that this operon was transcribed from ilvA through ilvE (i.e., ilvOADE). Favre et al. (9) showed that the valine resistant phenotype of the ilvO- mutation was due to the expression of a valine-insensitive isozyme of acetohydroxy acid synthase (isozyme II), the product of the normally cryptic ilvG gene. These workers mapped ilvG between ilvD and ilvE (i.e., ilvOADGE).

Recently this picture of ilv gene organization has been even further altered. It now appears that ilvO lies prior to ilvE, ilvG lies prior to ilvO and the direction of transcription of these genes is from ilvE through ilvA (i.e., ilvGOEDA) (3,5,7,20,21). It has not been determined whether ilvG is a spearate unit of transcription or is part of an ilvGEDA operon. Conflicting reports concerning this question have appeared in the literature (19,21,24).

All of the information currently available on the mode of regulation of the ilvEDA operon suggest that it is regulated by an attenuator mechanism (4,12,21).

We present physical evidence here that ilvE is indeed not the first structural gene of this operon and that it is preceded by another structural gene. We further show that it is unlikely that there is a regulatory region immediately prior to the ilvE gene and that "leader-like" RNAs are formed from DNA sequences common to two plasmids which share the ilv regulatory region. These data therefore suggest an ilvGEDA operon preceded by an attenuator.

MATERIALS AND METHODS

(a) Materials

Restriction enzymes either were obtained from New England Biolabs, were prepared by standard methods, or were a gift of Dr. Charles Yanofsky. E. coli DNA dependent RNA polymerase was a generous gift of Dr. Carl Parker. Alkaline phosphatase was obtained from Worthington Biochemical Corporation. T4 polynucleotide kinase was obtained from PL Biochemicals, Inc. T4 DNA ligase was obtained from Miles Laboratories. DNA polymerase A, Klenow fragment, was obtained from Boehringer Mannheim, Inc.

(b) Preparation of plasmid DNA

pLC26-3 was obtained from Dr. John Carbon. pRL5 was constructed by digestion of the h80dilv phage with Hind III restriction endonuclease. The phage restriction fragments were then cloned into the Hind III site of

pBR322 and individual colonies were subsequently screened by digestion with restriction endonuclease. Plasmid DNA was prepared by growing one litre cultures of the appropriate strains of L-broth to 150 Klett units (100 Klett unites equals 3 x 10^8 cells per ml;using a number 54 Green filter). 100 mg chloramphenicol was added to each culture and incubated for an additional 8-12 hr. Cultures were harvested by centrifugation and washed with 50 mM Tris-HCl, pH 8.5, 100 mM NaCl, 10 mM EDTA. The pellet was resuspended in 50 ml ice cold 50 mM Tris-HCl, pH 8.0, 25% sucrose. 10 ml of 250 mM Tris-HCl, pH 8.0, 5 mg/ml lysozyme (Sigma) were added and the suspension was swirled gently on ice for 5 min. This was followed by the addition of 20 ml of 250 mM EDTA, pH 8.0, after which the cell suspension was incubated on ice for 5 min. Immediately after the addition of 10 µl of heat inactivated bovine pancreatic ribonuclease A (2 mg/ml) in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA), 25 ml of Triton X100 (0.3% v/v) in 150 mM Tris-HCl, pH 8.0, 180 mM EDTA, was added and the incubation, on ice, was continued for 15 min. Cell debris and the E. coli nucleosome were removed by centrifugation at 35,000 x g for 1 hr. The supernatant was decanted and one-half volume of 30% polyethylene glycol (molecular weight 6,000) in 1.5 M NaCl was added (11). Plasmid DNA was allowed to precipitate overnight at 4°C. The pellet was collected by centrifugation for 3 min at 1,100 x g, and redissolved in 15.2 ml of 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 5 mM EDTA. CsCl, 15 g, were added, along with 0.4 ml of ethidium bromide (10 mg/ml), to the resuspended DNA. The refreactive index was adjusted to 1.390 and the plasmids were banded by centrifugation at 150,000 x g for 48-72 hr at 20°C. The plasmid band was collected and the ethidium bromide removed by isopropanol extraction. The DNA was dialyzed against 3 changes of 15 mM sodium citrate, pH 7.0, 150 mM NaCl, 1.0 mM EDTA. After dialysis, the DNA was concentrated by ethanol precipitation and resuspended in 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA.

(c) Purification of restriction fragments

Restriction endonuclease fragments were purified by polyacrylamide gel electrophoresis. 5% polyacrylamide (4.83% acrylamide, 0.17% bis-acrylamide) TBE (90 mM Tris, 90 mM H_3BO_3, 2.5 mM EDTA) gels were subjected to electrophoresis at 200-300 volts. The DNA fragments were visualized by staining with ethidium bromide and subsequently excised and eluted as described by Maxam and Gilbert (14).

(d) Enzyme reactions

Threonine deaminase was assayed as described previously (12). RNA

transcription was performed as described by Lee and Yanofsky (13), with 2 µg of RNA polymerase and with the substitution of 10 mM β-mercaptoethanol for dithiothreitol. After a 30-minute incubation at 37°C, the reaction was terminated by the addition of an equal volume of 2X TBE, 0.1% sodium dodecyl sulfate, 0.05% bromphenol blue, 0.05% xylene cylanol F F and 1.1 g urea per ml, and heated at 95°C for 2 min. Samples were subsequently subjected to electrophoresis on a 6% polyacrylamide, 7 M urea TBE gel at 200-300 volts.

The 5' phosphates of the DNA were labeled using $\gamma[^{32}P]$-ATP. The DNA fragment was prepared as described, with alkaline phosphatase present during restriction endonuclease digestion. The fragment was dried under vacuum and resuspended in 10 µl kinase denaturation buffer (10 mM glycine, 1 mM spermidine, 0.1 mM EDTA, pH 9.5) and incubated at 95°C for approximately 1 min. After chilling in an ice water bath, 2 µl of T4 polynucleotide kinase (2.8 units/µl) was added. This mixture was transferred to a silanized glass tube containing 1 µl of 10X kinase buffer (500 mM glycine, 100 mM $MgCl_2$, 50 mM dithiothietol, 50% v/v glycerol pH 9.5) and 150 µCi $\gamma[^{32}P]$-ATP (>3,000 Ci/mmol) previously dried under vacuum. The kinase reaction was performed at 37°C for 1 hr. The reaction was stopped by heating at 65°C for 15 min. Fragments were labeled at the 3' terminus by "filling-in" restriction sites with $\alpha[^{32}P]$-dATP (300 Ci/mmol). The fragments were resuspended in 29 µl H_2O, and 10 µl of polymerase buffer (500 mM Tris-HCl, pH 7.5, 100 mM $MgSO_4$, 10 mM dithiotheitol, 500 µg/ml bovine serum albumin); deoxynucleoside triphosphates without dATP were added to 1 mM, followed by 1 µl of the Klenow fragment of DNA polymerase A (10 units/µl). This mixture was transferred to a silanized glass tube in which 35 µCi $\alpha[^{32}P]$-dATP (300 Ci/mmol) had been dried under vacuum. The mixture was incubated at 20°C for 30 min. The reaction was terminated by heating at 65°C for 10 min.

(e) DNA sequencing

The DNA sequencing was performed as described by Maxam and Gilbert (14).

(f) Recombinant DNA statement

The recombinant DNA constructions reported here were performed according to standard microbiological procedures as prescribed by the current NIH guidelines for recombinant DNA research.

RESULTS

I. Localization of the *ilvGEDA* regulatory region

To establish the location of the regulatory region for the *ilvEDA*

genes we have utilized two plasmids, pRL5 and pLC26-3, which contain overlapping bacterial sequences. pRL5 was constructed by cloning the 4.8 Kilobase Hind III fragment from the λ h80dilv phage (Figure 1; ref. 15) into the Hind III site of pBR322. pLC26-3, obtained from Dr. John Carbon, is from a plasmid bank constructed by random shearing of the E. coli genome and insertion of the sheared fragments into the RI restriction site of the Col El plasmid by polynucleotide transferase tailing (6). This plasmid includes DNA sequences which complement each of the ilvEDA genes (data not shown). pLC26-3 also contains the DNA for the regulatory region of these genes since the synthesis of threonine deaminase (the product of the ilvA gene) on this plasmid responds to multivalent repression by leucine, isoleucine, and valine (Table I). The extent of the overlap of pRL5 and pLC26-3 is also indicated in Figure 2. The Hae III endonuclease restriction site most distant from the ilvE gene is the last known common restriction site

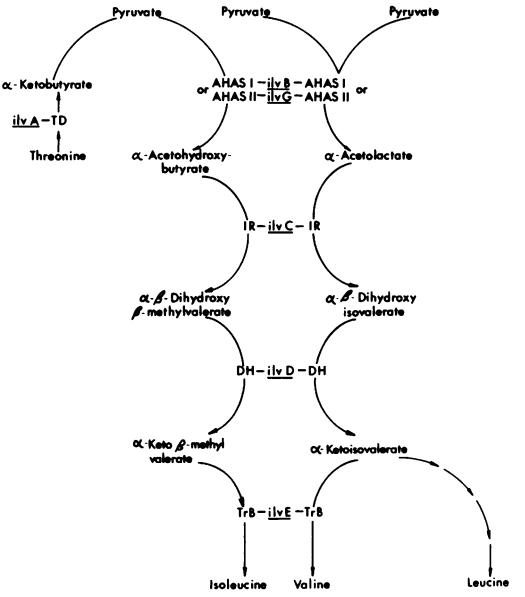

Figure 1 - Biosynthesis of isoleucine and valine. The enzymes involved in the biosynthesis of both isoleucine and valine are: TD, threonine deaminase; AHAS I, valine sensitive acetohydroxy acid synthase; AHAS II, valine resistant acetohydroxy acid synthase; IR, acetohydroxy acid isomeroreductase; DH, dihydroxy acid dehydrase; TrB, transaminase B. The ilv genes are tightly clustered on the E. coli chromosome at minute 83 (2) in the order ilvCADEG. The ilvGEDA operon is multivalently repressed by isoleucine, valine and leucine.

TABLE I

REGULATION OF THREONINE DEAMINASE ON pLC26-3

Strain	Threonine Deaminase, [a]Specific Activity		
	[b]Minimal	Minimal + LIV	L-Broth
[c]CU625	-	[d]b.d.	b.d.
CU625/pLC26-3	0.51	0.26	0.20

[a] Specific activity is expressed as μmoles α-ketobutyrate/min/mg protein.
[b] Minimal medium employed is M63 (Miller, 1972). Minimal + LIV is M63 supplemented with 0.5 mM leucine, 0.5 mM isoleucine, and 1.0 mM valine.
[c] CU625 contains the ilvE2070:Mu 1 polar mutation as described by Smith et al. (1976).
[d] b.d., below detection.

within the E. coli sequences contained on these two plasmids. This is illustrated in Figure 3, which shows a 5% polyacrylamide gel of Hae III restriction endonuclease digests of pLC26-3 (Lane 1), pRL5 (Lane 2), pBR322 (Lane 3) and a mixed Hae III, Kpn I restriction digest of pRL5 (Lane 4). Comparison of Lanes 1 and 2 with Lane 4 of Figure 2 shows that the 1.7 Kilobase fragment at the top of Lanes 1 and 2 contains the single Kpn I restriction site common to both plasmids. By comparing Lanes 1, 2, and 3, it can be seen that the only other readily detected Hae III fragment which is common to both pLC26-3 and pRL5 but not pBR322 is the 165 base pair Hae III fragment lying between the Kpn I site and Sal I site. Since the largest fragment found only in the Hae III restriction digest of pRL5 is approxi-

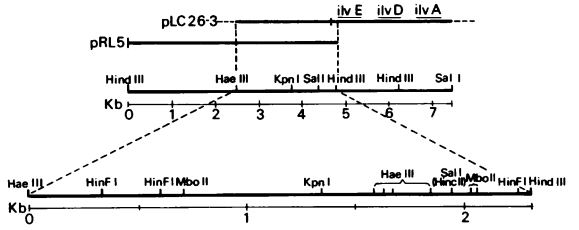

Figure 2 - Restriction endonuclease map of the ilvEDA region of E. coli K12 (Kb, Kilobases).

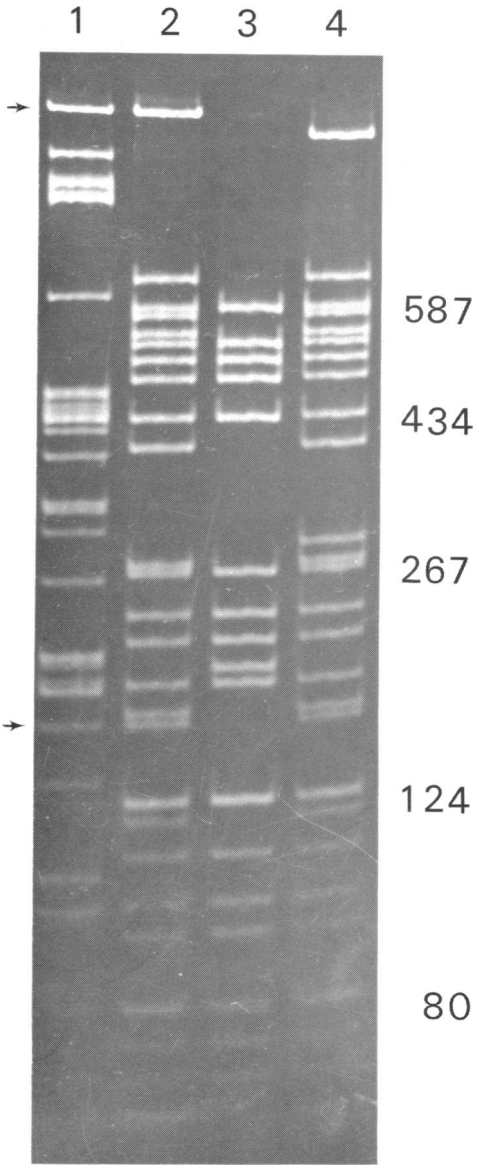

Figure 3 - Polyacrylamide gel electrophoresis of Hae III restriction endonuclease digestions of pLC26-3 (Lane 1), pRL5 (Lane 2), pBR322 (Lane 3) and a Kpn I, Hae III mixed restriction digest of pRL5 (Lane 4). Plasmids were digested at 37°C in 6 mM Tris-HCl, pH 7.6, 6 mM NaCl, 6 mM $MgCl_2$, 6 mM β -mercaptoethanol and subjected to electrophoresis on 5% polyacrylamide gels as described in Materials and Methods. Fragment lengths (base pairs) for pBR322 are according to Sutcliffe (23).

mately 600 base pairs, the remaining sequences common to these two plasmids must be less than 600 base pairs. Thus, the overlapping E. coli sequences contained on pLC26-3 and pRL5 are probably not more than 2.5 Kilobases in length.

II. DNA sequence preceding ilvE

Figure 4 presents the DNA sequence of the 574 base pair region prior to and including the first 220 base pairs of the ilvE gene. The beginning of the ilvE gene was established by comparison of the amino terminal amino acid sequence of the ilvE gene product, transaminase B (1,17), with the DNA sequence. The DNA sequence agrees with the first ten amino terminal residues of transaminase B (Gly-Thr-Lys-Lys-Ala-Asp-Tyr-Ile-Trp-Phe; ref. 1) except for the amino terminus which is glycine instead of threonine. Since the DNA sequence of the two independent plasmids pLC26-3 and pRL5 are identical (Lawther and Hatfield unpublished observations) and since two independent determinations in two separate laboratories yield the identical amino terminus

Figure 4 - DNA sequence of the region prior to and including the beginning of the ilvE gene. Coordinates are given in base pairs from the first base pair of the ilvE protein. The Sal I site is at -111 to -116 base pairs. The sequence AAGGA from -9 to -13 is complementary to the 3' terminus of 16S ribosomal RNA. The proposed terminus for ilvG is at -100 to -102 base pairs.

for transaminase B (1,17), it appears that the amino terminus of transaminase B must be post-translationally modified. This novel type of modification could be any which results in a modified threonine appearing as a glycine residue in the protein sequencing procedure. It should also be noted that the first five amino acids of transaminase B purified from Salmonella typhimurium (19) are identical to the amino terminus determined from the E. coli DNA sequence.

III. In vitro transcription of the ilvGEDA regulatory region

The attenuator model of gene regulation predicts the formation of a leader RNA by DNA dependent RNA polymerase initiating transcription at the promoter and terminating transcription at the attenuator site (4). The in vitro transcription of the 570 base pair Hpa II fragment from the operator-promoter region of the E. coli tryptophan operon yields two small RNA's. One is the 140 base leader transcript, while the second is the 260 base run-off transcript formed by RNA polymerase transcribing through the attenuator to the end of the Hpa II restriction fragment (Ref. 13; Figure 5, Lane 4). Comparison of the in vitro transcription of the Col El plasmid with that of the pLC26-3 and pRL5 shows that two leader-like RNA's are formed from the DNA sequence common to these two plasmids (Figure 2). Analysis of these in vitro transcripts by polyacrylamide gel electrophoresis shows three small RNAs (Figure 5, Lanes 2 and 3). Comparison of these lanes to the in vitro transcription of the Col El plasmid (Figure 5, Lane 1) shows that the smallest of these RNAs originates from sequences common to the two parental plasmids (Col El and pBR322). Comparison of the mobility of the two transcripts common to just pLC26-3 and pRL5 with the tryptophan leader and run-off transcripts indicates that the "ilv" specific transcripts are approximately 180 and 250 nucleotides in length. At present we cannot definitively explain the unexpected existence of two transcripts from this region.

DISCUSSION

The ilvE, D, and A genes have appeared to be an operon which is transcribed from ilvE through ilvA (5,20,24). This, combined with what have been considered to be cis dominant regulatory mutations, ilvO$^-$, (18,21) that map prior to ilvE (3,7,18,20,21,24), indicated that the regulatory region for this operon was adjacent to ilvE. Therefore, in order to obtain direct information about the gene organization of this operon, the location of the ilv regulatory region, and the nature of the regulation exerted on this operon,

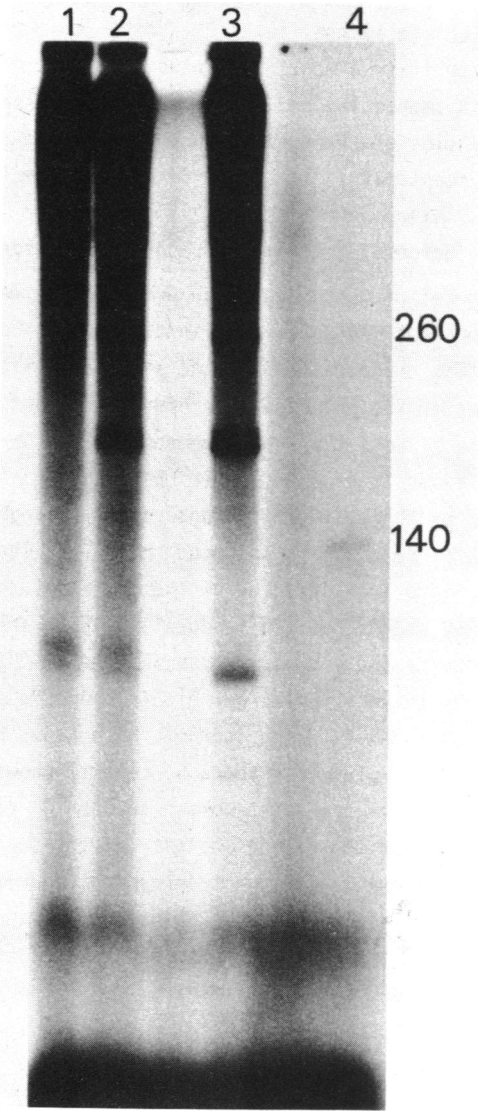

Figure 5 - Analysis of in vitro transcription of pLC26-3 and pRL5, RNA transcription was as described in Materials and Methods. Lane 1 contains the products of the in vitro transcription of the Col El plasmid. Lane 2 contains the transcription products of pLC26-3. Lane 3 contains the transcription products of pRL5. Lane 4 contains the transcription products of the 570 base pair Hpa II fragment from the trp operator-promoter region (13).

we sequenced the DNA preceding and including the beginning of the ilvE gene. These data reveal that ilvE is not the first gene of this operon but that it is preceded by another structural gene. This conclusion is based on the fact that there exists a translation termination site at -100 base pairs and that the translation frame preceding this site is in phase, without encountering another termination codon, for 232 base pairs (Figure 4). In fact, this translation frame is open for at least 826 base pairs (Lawther and Hatfield, to be published elsewhere). Although an unknown function could be postulated for this "gene," it seems more prudent to assume that this is the ilvG gene (3). We feel confident about this interpretation since we have recently obtained the DNA sequence of an attenuator region immediately preceding this gene which encodes a leader RNA containing multiple isoleucine, valine, and leucine codons (Lawther and Hatfield, submitted to Proc. Natl. Acad. Sci. U.S.A.). Further experiments to physically establish that this is, in fact, the ilvG gene are in progress. The significance, if any, of other shorter translation frames within this region are unknown.

The sequence data presented here also make it possible to understand several heretofore confusing facets of the regulation of this operon. Most importantly, it is now clear that the regulatory region which controls the expression of the ilvE, D, and A genes lies before the ilvG gene and that the region immediately preceding the ilvE gene is not a regulatory site. It now also appears clear that the ilvO$^-$ mutation relieves a site of natural polarity (22) within the ilvG structural gene. The ilvO mutation must therefore result in a loss of the polarity effect on the ilvEDA genes and allow for the translation and the expression of the entire ilvGEDA polycistronic message. We are currently sequencing the ilvG DNA from several ilvO strains in order to confirm this hypothesis.

The demonstration that leader-like RNAs are transcribed from the DNA region preceding ilvE further supports earlier suggestions that the ilvEDA genes are regulated by an attenuator mechanism. Preliminary data from this laboratory further shows that the 180 nucleotide leader RNA transcript specifically hybridizes to a 300 base pair restriction fragment present in both the pRL5 and pLC26-3 plasmids which encodes the ilv attenuator region prior to ilvG. At present we are not certain of the origin of the larger 250 nucleotide transcript.

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