Cloning, Expression, and Nucleotide Sequence of *livR*, the Repressor for High-Affinity Branched-Chain Amino Acid Transport in *Escherichia coli*

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ABSTRACT   The *livR* gene encoding the repressor for high-affinity branched-chain amino acid transport in *Escherichia coli* has been cloned from a library prepared from the episome F106. The inserted DNA fragment from the initial cloned plasmid, pANT1, complemented two independent, spontaneously derived, regulatory mutations. Subcloning as well as the creation of deletions with Bal31 exonuclease revealed that the entire regulatory region is contained within a 1.1-kb RsaI-SalI fragment. Expression of the pANT plasmids in *E. coli* minicells showed that the regulatory region encodes one detectable protein with an apparent molecular weight of 21,000. DNA sequencing revealed one open reading frame of 501 bp encoding a protein with a calculated MW of 19,155. The potential secondary structure of the regulatory protein has been predicted and it suggests that the carboxy terminus may fold into three consecutive alpha helices. These results suggest that the *livR* gene encodes a repressor which plays a role in the regulation of expression of the *livJ* and the *livK* transport genes.

Key words: regulation, prokaryotic bacteria, leucine uptake, leucyl tRNA corepressor, cell physiology

INTRODUCTION

Branched-chain amino acid transport in *Escherichia coli* is carried out by three distinct transport systems. The system designated LIV-I transports the L-isomers of leucine, isoleucine, and valine with a high affinity and threonine and alanine with a somewhat lower affinity.[1] The leucine-specific (LS) transport system transports primarily the D- and L-isomers of leucine with relatively high affinity.[1] These two high-affinity systems are regulated in response to the intracellular levels of leucine. The third transport system, LIV-II, operates with a low affinity for leucine, isoleucine, and valine and is not significantly regulated by any of the branched-chain amino acids.[2]

The LIV-I and LS transport systems have been genetically mapped to minute 76 on the *E. coli* chromosome.[3,4] The genes encoding the protein components of these two transport systems have been cloned,[5] and it appears that five proteins are required to effect transport. The LivH, LivM, and LivG proteins are components of both the LIV-I and LS transport systems, and these components appear to be associated

with the inner membrane.[6-8] The *livK* gene product (LS-BP) is a periplasmic binding protein [9] component of the LS system that utilizes both the D- and L-isomers of leucine as substrates. The *livK* gene is the first gene of an operon which includes the *livH*, *livM*, and *livG* genes in that order.[10,11] The LIV-I transport system utilizes a periplasmic binding protein (LIV-BP) encoded by the *livJ* gene.[12] The *livJ* gene is transcribed from a monocistronic operon and, together with the *livH*, *livG*, and *livM* genes, encodes the components of the LIV-I transport system.[10,11]

A previous report[2] described two strains of *E. coli*, each harboring an independent, spontaneous regulatory mutation for branched-chain amino acid transport. Each of these mutant strains exhibited derepressed rates of L-leucine transport, even in the presence of high concentrations of leucine. One of these mutations, *livR*, affected both the LIV-I and LS transport systems, whereas the *lstR* mutation affected primarily the LS transport system. The *livR* and *lstR* mutations are closely linked at minute 20 on the *E. coli* chromosome.[13] Since these regulatory loci are unlinked to the transport genes, it was deduced that they encode a diffusible regulatory component. Further studies showed that the episome F106 complements each mutation by restoring leucine regulation of LIV-I and LS transport.[13]

In order to determine the number and nature of the regulatory gene(s) for the LIV-I and LS transport systems, a DNA library in plasmid pBR322 was constructed from the partially digested episome F106 and a single clone containing the regulatory region was identified. This article describes the characterization and expression of the regulatory gene for branched-chain amino acid transport.

MATERIALS AND METHODS
Bacterial Strains

The *E. coli* strain DH-1 ([F−,λ−] *recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17* [r_K-, m_{K+}], *supE44*,) was used for all initital recombinant plasmid constructions. Strains AE62, AE68(*livR*), AE79 (*livR*, *leu*), and

AE117 (lstR, leu, dlu) were described previously.[13] The E.coli minicell-producing strain X1411 (minA1, minB2, supE42) was a gift from Dr. R. Helling of the University of Michigan. Strain JM101[14] was used in DNA sequencing.

Materials

MOPS (morpholinopropane sulfonic acid), nutritional supplements, γ-(methacryloxy)-propyltrimethoxysilane (M-6514), and the Dalton Mark VI molecular weight markers for denaturing polyacrylamide gel electrophoresis were purchased from the Sigma Company. Aqua-Sil was from the Pierce Chemical Company and the DNA Grade Bio-Gel HTP was from BioRad Laboratories. Bactotryptone, bacto-agar, and yeast extract were products of the Difco Company, Detroit, Michigan. Restriction enzymes, M13 phage, X-gal, and isopropylthio-β-D-galactoside were obtained from Betheseda Research Laboratories. The T4 DNA ligase was from International Biotechnologies, Inc. and the calf intestine alkaline phosphatase was obtained from the Boehringer Mannheim Company of W. Germany. The L-[^{3}H]-leucine, L-[^{35}S]-methionine, α-[^{35}S]-dATP, γ-[^{35}S]ATP, Klenow polymerase, T4 polynucleotide kinase, M13 universal primer, and Colony/Plaque Screen hybridization transfer membrane were from New England Nuclear. The source of all nonradioactive dideoxy- and deoxynucleotides was Pharmacia P-L Biochemicals.

Media

Standard LB media[15] supplemented as required with thymine (50 μg/ml) or ampicillin (30 μg/ml) was used to select transformants. For transport assays, Vogel-Bonner (VB) minimal media[16] was used and supplemented with L-leucine at 25 μg/ml when indicated. Growth of minicells was done in MOPS rich media.[17] For incorporation of L-[^{35}S]-methionine, MOPS-rich media without methionine was used. Strain JM101 was grown on 2×YT media or M9 minimal media which were made as described previously.[14]

Transport Assays

Cells were grown in 1-ml cultures overnight at 37°C in VB media. For strains AE62 (control) and AE68 (livR), cultures were grown with and without 25 μg/ml L-leucine. Strain AE117 (lstR) was a leucine auxotroph and consequently was always grown in VB media supplemented with leucine. If the cells were harboring plasmids, ampicillin was included to select for retention of the plasmids. The overnight cultures were used to inoculate 4-ml cultures (1:10 dilution) which were grown to mid-log phase (OD$_{420}$ = 0.4–0.7). The cells were washed and resuspended in ice-cold, unsupplemented VB media so that their final OD$_{420}$ was between 0.4 and 0.8. Tubes containing 0.4 ml of VB media plus L-[^{3}H]-leucine at a final concentration of 200 nM were equilibrated to 37°C. Then,

0.4 ml of the cell culture (briefly equilibrated to 37°C) was mixed with the tritiated solution for 10 seconds, pipetted onto a hydrated 0.45-μm nitrocellulose filter, and immediately washed 2× with 5 ml of prewarmed 10 mM potassium phosphate buffer (pH 7.2) containing 0.1 mM MgSO$_4$. Radioactivity was determined by using a Packard liquid scintillation counter and standard scintillation cocktail. Leucine uptake was calculated as nmoles leucine/min/mg dry cell weight.

Construction of Recombinant Plasmids

The DNA from the episome F106 was prepared by growing cells to stationary phase and gently lysing with an SDS solution.[15] Two consecutive cesium chloride-ethidium bromide gradient separations were carried out by the method of El-Gewely and Helling.[18] The samples were then dialyzed against 4 liters of 10 mM Tris-HCl (pH = 8.0), 1 mM EDTA overnight. The F106 DNA was partially digested with EcoRI and the resulting fragments in the 3–10-kb range were purified by using low-melting agarose.[19] This mixture of fragments was ligated at 12°C overnight with pBR322 DNA which had been linearized with EcoRI and dephosphorylated by standard procedures.[15] The ligation reaction (approximately 0.5 μg of DNA) was transformed into DH-1 cells made competent by the protocol of D. Hanahan.[20] Ampicillin was used to select for colonies carrying recombinant molecules. These colonies were then grown to late log phase and the plasmid DNA was isolated by the rapid boiling method and transformed into strain AE68 (livR) made competent by CaCl$_2$ treatment.[15] Colonies resistant to ampicillin were tested by the transport assay described above carried out both in the presence and in the absence of leucine.

Isolation of Minicells

The minicell strain X1411 was rendered competent by the CaCl$_2$ method described above and was transformed with plasmids pBR322 (control), pANT1–pANT4, and pANT9. These cells were then grown in 1-ml cultures overnight at 37°C in MOPS-rich media plus ampicillin. Cultures of 250 ml were inoculated and grown until late log phase (OD$_{600}$=0.8–1.0). Minicells were isolated by a combination of differential centrifugation and differential-rate sedimentation through two consecutive sucrose gradients as described previously.[21] The minicells were then resuspended in the appropriate volume to yield an OD$_{600}$=0.5 per 400 μl. Aliquots of 400 μl were frozen with 10% (v:v) glycerol at −70°C. The ratio of whole cells to minicells was less than a maximum of 1:10^4.

In order to radioactively label proteins produced by the minicells, an aliquot of the minicell preparation was thawed and the cells were pelleted at 12,000g in a minicentrifuge for 5 minutes. The pellet was resuspended in 100 μl MOPS rich media without methionine. The cells were incubated for 20 minutes at 37°C in order to allow degradation of chromosome-encoded mRNAs carried over from the whole cells. One

hundred μCi of L-[^{35}S]-methionine were added to the cells and incubation was continued for 45 minutes. The cells were lysed by mixing with SDS running buffer and by boiling for 3 minutes. Electrophoresis on SDS-polyacrylamide gels[22] was carried out at 15 W until the bromophenol blue had reached the bottom of the gel. The gel was fixed for 60 minutes in 40% methanol/10% HOAc and subsequently dried under vaccuum onto Whatman 3MM paper. Autoradiography was carried out at least 12 hours at room temperature without intensifying screens.

Recombinant DNA Cloning and DNA Sequencing Strategies

Two strategies were used to sequence the DNA containing the wild-type regulatory region for the LIV-I and LS transport systems. One strategy involved directional cloning of the 1.8-SalI-KpnI fragment from pANT3 (see Fig. 2) in both orientations into the M13 vectors mp18 and mp19. The resulting clones were designated mpANT5 and mpANT6 and were then used as templates for sequencing with the M13 universal primer. Additional primers were synthesized based on the early sequence results in order to "walk" into the 2-kb fragment. Approximately 400 bp were sequenced by this method.

The other strategy used to deduce the wild-type sequence was to "shotgun" clone the 1.8-SalI-KpnI fragment by digesting it with TaqI or with Sau3A and by ligating the resulting pieces to mp10 or mp11 linearized with the restriction endonucleases AccI or BamHI, respectively. The majority of the regulatory region was sequenced from these clones. The remaining gaps in the sequence as well as the remaining unsequenced complementary strands were completed by utilizing synthesized oligonucleotides as primers.

Chain Elongation Reactions and Electrophoresis

The annealing of primer to template and the elongation reactions were carried out as described previously.[23] The denaturing gels were 8% acrylamide with a buffer gradient (2.5× at the bottom; 0.5× at the top) and were prepared as described previously.[24] In addition, one gel plate was treated with Aqua-Sil, which repels the acrylamide gel, while the other plate was treated with the γ-(methacryloxy)-propyltrime-thoxysilane, which covalently bonds the acrylamide gel to the glass.[25] The gels were electrophoresed at 20 mA (constant) until the bromphenol blue or the xylene cyanol reached the bottom of the gel. At this point, the plates were separated and the gel (which is bonded to one of the plates) was soaked twice in a solution of 10% HOAc: 10% methanol for 15 minutes. The gel bonded to the glass plate was dried at 80°C for 1–2 hr and autoradiography was carried out for 24–48 hr.

Oligonucleotide Synthesis and Purification

After the oligonucleotides were synthesized, 1 ml of fresh, cold concentrated ammonium hydroxide was added to 0.5× volume recovered from the column and the mixture was incubated overnight at 55°C. The oligo mix was then lyophilized and resuspended in 250 μl of 10 mM Tris-HCl, 1 mM NaEDTA (pH 8.0). Approximately 0.05× volume was used in an end-labeling reaction with T4 polynucleotide kinase and γ-[^{35}S]ATP. The radioactively labeled oligonucleotide was subjected to chromatography on a 3-ml G-25 column made in H$_2$O. Fractions of 100 μl were collected and 10% of each fraction was mixed with 4 ml of liquid scintillant in order to quantitate its radioactivity. The fractions containing the oligonucleotide were collected, pooled, and then used as primers in the DNA sequencing reactions.

Computer Analysis of the DNA Sequence

All analyses of the DNA sequences and secondary structure predictions were done by using an IBM PC-AT, a Hayes Smartmodem 1200, and the BioNet Computer Resource.

RESULTS

A DNA library was constructed as described in Materials and Methods and one of the cloned plasmids, pANT1, was found to contain sequences that complemented the *livR* mutation. Figure 1 shows the transport activities for several strains grown in the absence and presence of 25 μg/ml leucine. Strain AE62 is wild type with respect to regulation of leucine transport and demonstrates a four to five fold decrease in the rate of leucine uptake when grown in the presence of leucine. Strain AE68 (*livR*) exhibits a
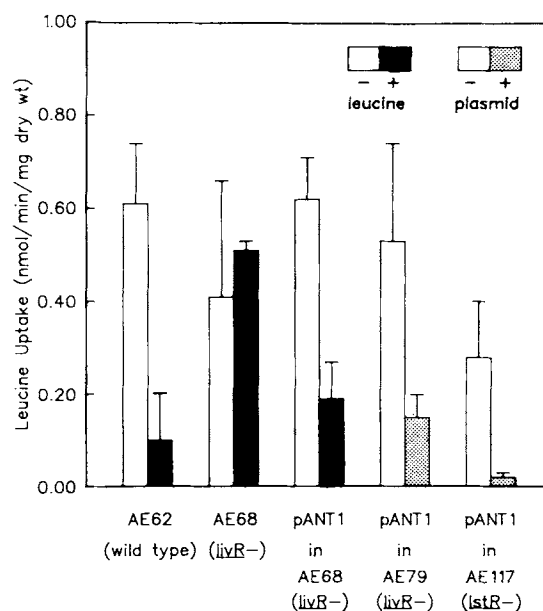

Fig. 1. Complementation of regulatory mutations by the plasmid pANT1. Strains were grown either in the absence (open bars) or presence (filled bars) of 25 μg/ml leucine in minimal media. For strains AE79 and AE117, both leucine auxotrophs, open bars represent the strains grown with leucine and without plasmids, while the stippled bars represent the strains carrying the plasmid pANT1 grown with leucine.
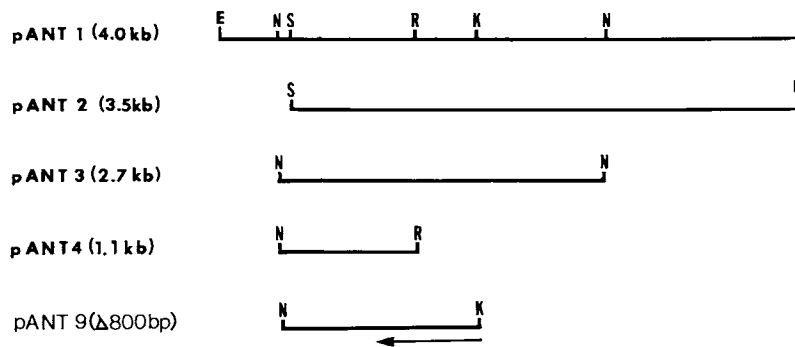
Fig. 2. Construction of subclones from the plasmid pANT1. The top line shows the insert (4.0 kb) of pANT1 and the following lines depict the inserts of the subclones. Abbreviations are as follows: E = EcoRI, S = SalI, N = NruI, R = RsaI ,K = Kpn. The arrow under pANT9 indicates the extent and direction of deletion by Bal31 exonuclease.
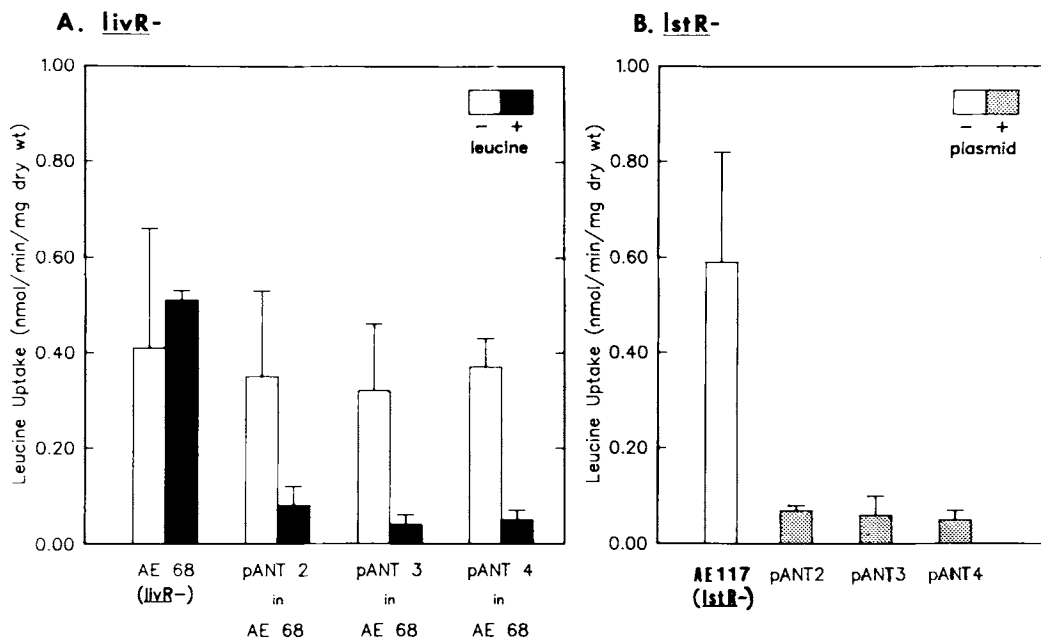

Fig. 3. Complementation of the regulatory mutations by subclones of the plasmid pANT1. Part A shows the rates of leucine uptake for strain AE68 (*livR*) harboring the pANT subclones. Part B shows the rates of leucine uptake for strain AE117 (*lstR*) carrying the pANT subclones.

constitutively derepressed rate of leucine transport; however, when this strain was transformed with the plasmid pANT1 and grown with leucine, the rate of leucine transport was repressed. In order to confirm this result, the plasmid pANT1 was transformed into strain AE79 (*livR, leu*). This strain shows a derepressed rate of leucine transport in the presence of leucine (open bar) which was significantly reduced when the plasmid pANT1 was present (stippled bar).

To determine whether the inserted fragment of the plasmid pANT1 also complemented the *lstR* mutation, the plasmid was transformed into strain AE117 (*lstR, leu, dlu*). Figure 1 shows the repression of leucine transport was achieved only when strain AE117 grown with leucine was harboring the plasmid pANT1. These data indicate that the recombinant plasmid pANT1 contains DNA sequences which can

genetically complement both the *livR* and *lstR* mutations; therefore the cloned DNA insert from pANT1 encodes the regulatory component for branched-chain amino acid transport.

Four subclones were constructed from the 4.0-kb insert of the plasmid pANT1 (Fig. 2). It was anticipated that if multiple regulatory genes existed, the above strategy would permit them to be separated and identified with these subclones. The plasmid pANT2 contained the 3.5kb EcoRI-SalI fragment from pANT1 ligated to EcoRI-SalI double-digested pBR322. The plasmid pANT3 was constructed from the 2.7 kb NruI fragment of pANT1 ligated with NruI-linearized pBR322. The third subclone to be discussed at this point, pANT4, was made by ligating the 1.2-kb RsaI-NruI fragment from pANT3 to NruI-linearized pBR322. As presented in Figure 3, the inserted frag-
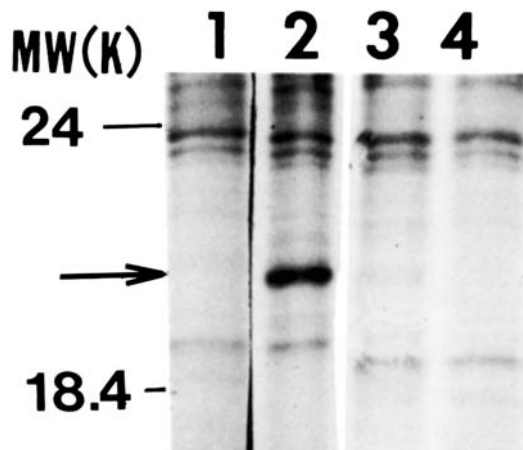
Fig. 4. In vivo expresssion of the pANT plasmids in *E. coli* minicells. This autoradiograph shows the proteins produced from *E. coli* minicells which have been separated by SDS-PAGE. The proteins have incorporated L-[^{35}S]-methionine and each minicell sample carries a different plasmid. For each sample, one-half of the volume of one minicell aliquot was loaded onto the gel. Lane 1, pBR322; lane 2, pANT3; lane 3, pANT4; lane 4, pANT9.
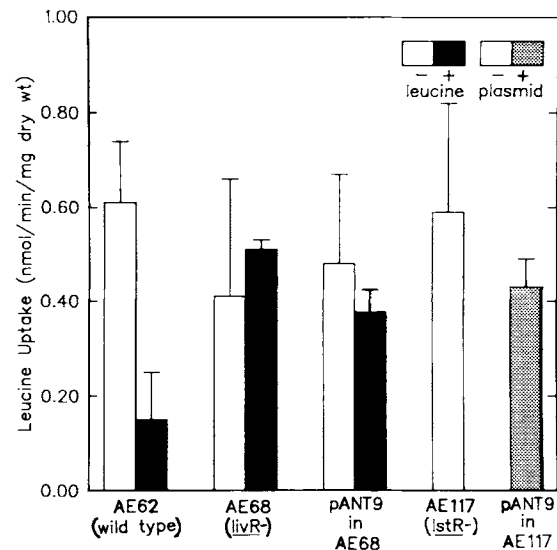

Fig. 5. Complementation of the regulatory mutations by the deletion plasmid pANT9. Strains AE62 and AE68 (*livR*) were grown either in the absence (open bars) or in the presence (filled bars) of 25 μg/ml leucine in minimal media. Strain AE117 (*lstR*) is a leucine auxotroph, so it was grown either with leucine and without plasmid (open bar) or with leucine while harboring the plasmid pANT9 (stippled bar).

ments of all three subclones complemented the *livR* and the *lstR* mutations. These results strongly suggest that the regulatory region resides within the 1.1-kb SalI-RsaI fragment.

The *E. coli* minicell strain X1411 was used with the plasmids pANT1–pANT4 and pBR322 (control) in an attempt to identify a potential gene product. Figure 4 represents an autoradiograph of two 15% SDS-polyacrylamide gels used to separate the protein products from the minicell samples. Lane 1 shows the proteins encoded by the plasmid pBR322 as well as the background products presumably from long-lived chromosome-encoded mRNAs contained in the minicells. Lanes 2 and 3 correspond to products from minicell samples harboring the plasmids pANT3 and pANT4, respectively. Samples that contained the plasmids pANT1 or pANT2 had banding patterns identical to the one for the plasmid pANT3 (data not shown). In each lane, the only detectable band correlating with the presence of plasmids pANT1–pANT4 but not with the plasmid pBR322 was the protein of molecular weight 21,000, which is indicated by the arrow.

It was observed that the expression of this protein from pANT4 was significantly and specifically less than that from the other plasmids (Fig. 4, lane 3). One possible explanation for this decreased expression was that some of the DNA sequence required for the wild-type level of expression may have been deleted during the construction of plasmid pANT4. It therefore seemed possible that the promoter region of the gene encoding the protein flanked the RsaI site used in the construction of the plasmid pANT4 and that the 3' end of the gene was near the SAlI site. Furthermore it could be deduced from its MW that the protein must be encoded by a gene approximately 500–600 bp in length. Since it was possible that two such genes could exist in the 1.1-kb SalI-RsaI frag-

ment, it could not yet be concluded that the protein of MW 21,000 was indeed the regulatory protein for leucine transport.

In order to determine the location of the regulatory gene(s) within the 1.1-kb SalI-RsaI fragment, a deletion plasmid, pANT9, was prepared by linearizing the plasmid pANT3 at the KpnI site (See Fig. 2), digesting the DNA with Bal31 exonuclease, repairing the DNA ends with Klenow polymerase, and ligating the resulting partially deleted fragments. The plasmids were screened by SalI digestion, and the deletion plasmid pANT9, missing a total of 1.5–1.6 kb, was chosen for further experiments. It was estimated by restriction mapping that the plasmid pANT9 had lost the RsaI site as well as an additional 100 bp of DNA (data not shown). While a deletion of this size was expected to abolish expression of the gene encoding the protein of MW 21,000, it would not be expected to interfere with the expression of other genes located downstream.

Figure 5 shows the rate of leucine uptake for strains AE68 (*livR*) and AE117 (*lstR*) harboring the plasmid pANT9 grown both in the presence and in the absence of 25 μg/ml leucine. The rate of leucine uptake for these two strains remained derepressed even in the presence of high concentrations of leucine, showing that the DNA sequences remaining in the plasmid pANT9 did not complement either regulatory mutation. Moreover, lane 4 of Figure 4 shows the [^{35}S]-labeled proteins produced from *E. coli* minicells containing pANT9, and it is clear that the protein of MW 21,000 is not detectable. These results suggest that this protein corresponds to the regulatory component for branched-chain amino acid transport.

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                                                   27                                        54
ATG GGC GTG TTC TCT TTG GTC TGG ATC TCC ATT GCC GGC GCG CAG GTA CGT CCG
MET Gly Val Phe Ser Leu Val Trp Ile Ser Ile Ala Gly Ala Gln Val Arg Pro


                                                   81                                       108
CGA TCA TTG CGC CGA ACA ACA GCG CGC GGT TCT TTC GCA ATA CAT AAG CAC GAA
Arg Ser Leu Arg Arg Thr Thr Ala Arg Gly Ser Phe Ala Ile His Lys His Glu


                                                  135                                       162
CAA GAA ACA GAT AAC CAC GCC TGC CAT AAC ACC ATC AAG CCG AAC TCT TCA CAT
Gln Glu Thr Asp Asn His Ala Cys His Asn Thr Ile Lys Pro Asn Ser Ser His


                                                  189                                       216
GCT TGG TGC ACC TGC GAA TGT AAC CGA AGG TCG GTG GTT AGC ACG CAT AAA GCC
Ala Trp Cys Thr Cys Glu Cys Asn Arg Arg Ser Val Val Ser Thr His Lys Ala


                                                  243                                       270
AGC ATA ATT CCG GCG AAT AAC AAC CCG GCA GCG TTC CCC ATG CCG AAA CTG AAT
Ser Ile Ile Pro Ala Asn Asn Asn Pro Ala Ala Phe Pro MET Pro Lys Leu Asn


                                                  297                                       324
GTG CTG AAC TGG AAG GTG ATC ATC CCG ATC ATC AGC CCA ATA ACA AAG AAG GCG
Val Leu Asn Trp Lys Val Ile Ile Pro Ile Ile Ser Pro Ile Thr Lys Lys Ala


                                                  351                                       378
CAG AAT GCC AGC AGG TCA GTG ACC TGG ACT GTG AAT CGA GAT AAA GCC GAT GCG
Gln Asn Ala Ser Arg Ser Val Thr Trp Thr Val Asn Arg Asp Lys Ala Asp Ala


                                                  405                                       432
ATC CAG TGT TTT TAC GCG GCG GGC ATC GTG GAT CTT GTA AAA GGT CAC TTT GTT
Ile Gln Cys Phe Tyr Ala Ala Gly Ile Val Asp Leu Val Lys Gly His Phe Val


                                                  459                                       486
AAG CAC GAC GTT GTC ATC TAT CGG CAT CTC AAT CTG GCT AGA ATG ACG CGG TTA
Lys His Asp Val Val Ile Tyr Arg His Leu Asn Leu Ala Arg MET Thr Arg Leu


                        513
AGG AAG CAA CCG TGA
Arg Lys Gln Pro  .
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Fig. 6. Complete DNA sequence of the coding region of the *livR* gene. The top line indicates DNA sequence and the bottom line is the translated amino acid sequence.

DNA sequencing of the regulatory region supports this interpretation since there is only one complete open reading frame within the inserted fragment from plasmid pANT4. Figure 6 shows the DNA sequence of the coding region of the *livR* gene along with its translated protein sequence. The gene is composed of 501 bp and encodes a protein of 166 amino acids. This predicted protein has a calculated MW of 19,155, which is in good agreement with the MW of 21,000 observed for the regulatory protein from the minicell expression system.

Figure 7 shows the amino acid sequence of the LivR protein and beneath are designated the predicted alpha helices (A), beta sheets (B), and turns (T). It is possible that the carboxyl terminus of the protein consists of a helix-turn-helix-turn-helix conformation.

The promoter region of the *livR* is presented in Figure 8. Three RsaI sites (5'-GTAC-3') are visible on this figure at positions −200, −192, and −45. From the expression studies carried out on the pANT4 subclone it was deduced that the entire gene and a functional promoter were encoded downstream from the RsaI site at −45. In this region, the only ATG followed by a significant open reading frame is found at

the −2 position. There is a potential Shine-Dalgarno ribosome binding site[26] from position −10 to position −6. A strong promoter (i.e., Pribnow box and −35 region)[27] was detected between positions −23 and −55. According to the algorithm deduced by McClure and his associates, [28,29] an ineffective promoter has a score of 45 or less while a very strong promoter may score as high as 80. With the aid of this algorithm, the potential promoter sequence from −23 to −55 has a score of 66, which suggests that this is probably a relatively strong in vivo promoter. Another potential promoter region is observed between positions −62 and −103. Although the expression of *livR* from pANT4 indicated that this cannot be the sole promoter for *livR*, its score with the McClure algorithm was 62, which is very similar to that of the first promoter. Thus, based on sequence homologies, there are two regions which are equally likely to drive *livR* gene expression.

DISCUSSION

Previous reports on high-affinity branched-chain amino acid transport in *E. coli* revealed that two operons regulated by the intracellular levels of leu-

cine encode the protein components of the LIV-I and LS transport systems. Each of these operons, located at minute 76 on the *E. coli* chromosome, has a distinct *cis*-acting regulatory region. Moreover, independent, *trans*-acting regulatory mutations have been identified for each transport operon. These two mutations (*livR* and *lstR*) were mapped to minute 20 on the *E. coli* chromosome and were not separable from each other genetically.

We, therefore, attempted to clone the minute 20 region from *E. coli*, and in this report we show that
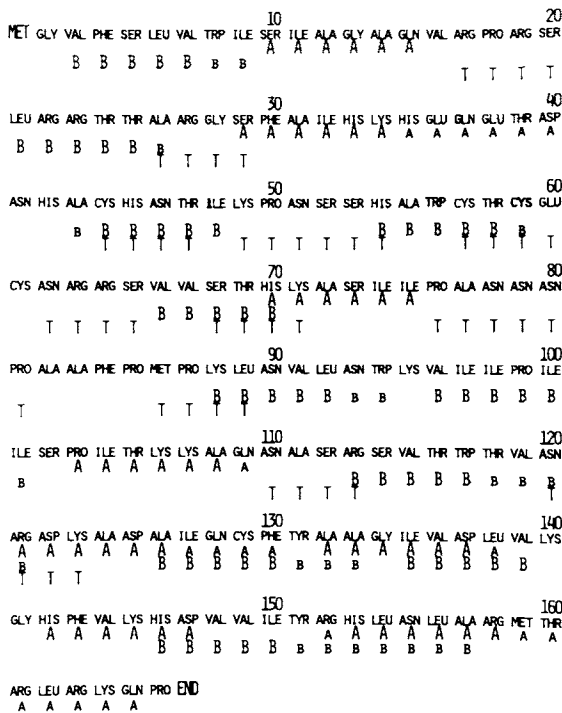

Fig. 7. Predicted secondary structure of the LivR protein. This figure shows the translated amino acid sequence and below it, the secondary structure predictions. The legend is as follows: A, alpha helix; B, beta sheet; and T, turn. Big letters indicate a strong propensity for that residue to conform to the predicted structure while small letters indicate a weaker probability for the predicted structure. The secondary structure predictions were generated by the PEP program of BioNet. This program uses the Chou and Fasman method.

the gene encoding the regulatory component for high-affinity branched-chain amino acid transport has been isolated in the recombinant plasmid pANT1. Four subclones have been constructed and three of these plasmids (pANT2, pANT3, and pANT4) complement the two regulatory mutations (*livR* and *lstR*) and express a protein with an apparent MW of 21,000. A fourth subcloned plasmid, pANT9, carries a deletion which results in no detectable expression of the protein of MW 21,000. Concomitantly, this deletion subclone does not restore regulation of leucine transport in mutant strains containing either the *livR* or the *lstR* mutation. These data strongly suggest that the gene which encodes the protein of MW 21,000 is also the regulatory gene for high-affinity branched-chain amino acid transport.

Several pieces of evidence indicate that the LivR protein is probably a standard prokaryotic repressor. The *livR* gene is unlinked to the LIV-I and LS transport operons so that it is *trans*-acting; moreover, genetic studies demonstrated that the presence of functional LivR protein repressed the transport genes' expression. The size of the LivR protein is within the size range of other prokaryotic and phage repressors (MW of cro protein is 8000,[30,31] MW of lambda repressor is 25,000,[32] and the MW of the trp repressor is 12,500 [33]). The predicted secondary structure of the LivR protein includes a potential helix-turn-helix-turn-helix conformation which is common to many DNA-binding proteins, including repressors. However, although it seems most likely that the LivR protein is a repressor, it should be noted that the LivR protein may also be a regulatory factor involved in attenuation of transport gene expression.

It is asssumed that the liv repressor utilizes a corepressor, but its identity has not been determined. Since the liv repressor functions in response to the intracellular levels of leucine, one likely candidate for the corepressor is leucine itself. However, there is significant genetic evidence implicating charged leucyl-tRNA in the regulation of the rates of transport. In strains carrying the *hisT* mutation[34] (pseudo-uridine in position 41 of the leucyl-tRNA) or the *leuS* mutation[35] (temperature sensitive leucyl-tRNA), the rate of branched-chain amino acid transport is consti-
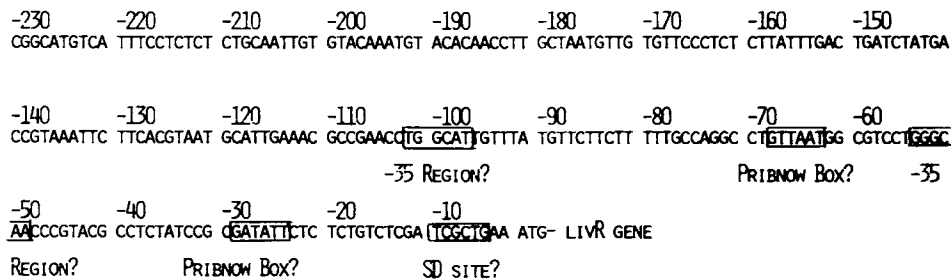

Fig. 8. Promoter sequence of the *livR* gene. Shown are 230 bp of the 5' noncoding region of the *livR* gene. A probable Shine-Dalgarno consensus site is located at – 10. There are two potential promoters (positions – 23 to – 55 and – 62 to – 103).

tutively derepressed. Thus, another potential candidate for the liv corepressor is the charged leucyl-tRNA.

Interestingly, since there is enough liv repressor expressed in the *E. coli* minicells to be stained with Coomassie Brilliant Blue R (data not shown) and since the inserts of plasmids pANT1, pANT2, and pANT4 are oriented such that *livR* gene expression is not likely to be driven by either one of the plasmid promoters, this suggests that the liv repressor gene has a relatively strong endogenous promoter. This deduction is supported by the presence of potential tandem promoters as determined from the DNA sequence.

Earlier studies have shown that leucine appears to play an important role in bacterial cell physiology. For example, in *E. coli*, leucine is the only branched-chain amino acid that serves to regulate LIV-I or LS transport;[12] leucine is one of the amino acids to which bacteria exhibits sensitivity;[36] and leucine represses activity of several operons.[37-40] Leucine also has been shown to stimulate the expression of several operons in *E. coli*.[38,41-43] A detailed understanding of how leucine regulates its own transport activity will be necessary to understand its central role in the physiology of the bacterial cell. A plausible mechanism by which leucine may control other operons could involve the liv repressor. Studies are underway to determine its role in the regulation of leucine transport.

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