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Premature Termination of In Vivo Transcription of a Gene Encoding a Branched-Chain Amino Acid Transport Protein in *Escherichia coli*

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Previous studies have suggested that control of expression of genes of the LIV-I permease system for the high-affinity transport of branched-chain amino acids in *Escherichia coli* involves modulation in the frequency of mRNA elongation. Mutation of the Rho transcription termination factor and shortages of charged leucyl-tRNA have been shown to alter LIV-I transport activity. Rho-dependent transcription termination regulated by shortages of charged leucyl-tRNA at sites preceding structural genes has been proposed to account for their role in regulation of LIV-I transport. Transcription of the *livJ*-binding protein gene, encoding one of the periplasmic components of the LIV-I system, was analyzed in vivo with strains which lack repression of the LIV-I genes and harbor a temperature-sensitive allele for either leucyl-tRNA synthetase or Rho factor. Analysis of mRNA synthesis by DNA-RNA hybridization in the various mutant strains indicated that both shortages of leucyl-tRNA caused by inactivation of the temperature-sensitive leucyl-tRNA synthetase and inactivation of the Rho factor were associated with increased synthesis of *livJ* mRNA. Nuclease protection and gel electrophoresis studies detected prematurely terminated transcripts corresponding in size to the leader region of *livJ* mRNA. Accumulations of these short transcripts were suppressed in strains harboring temperature-sensitive alleles for either leucyl-tRNA synthetase or Rho factor. These results provide support for the hypothesis that expression of *livJ* involves Rho-dependent transcription termination in which antitermination is associated with the intracellular availability of aminoacyl leucyl-tRNA.

The LIV-I permease system mediates the high-affinity transport of leucine across the inner membrane of *Escherichia coli* by interactions of periplasmic binding proteins with a set of membrane-associated proteins (19). Genes for the periplasmic and membrane proteins lie in a cluster at 76 min on the *E. coli* chromosome map (2). Translation products of *livH*, *livM*, *livG*, and *livF* form the membrane-associated proteins. The leucine-specific and general branched-chain amino acid-binding proteins are the products of *livK* and *livJ*, respectively. Genes for *livK*, *livH*, *livM*, *livG*, and *livF* are organized into a single transcription unit, whereas *livJ* lies approximately 1 kb upstream of the *livKHMGF* gene group (1).

Synthesis of the periplasmic and membrane-associated proteins of the LIV-I system is repressed by growth of *E. coli* at high levels of leucine (26). The predominant mode for regulation of synthesis is thought to be control of transcription initiation. A *trans*-acting regulatory locus, designated *livR*, is thought to encode a regulatory factor effecting repression control of the LIV-I genes (3). The *livR* locus might be an allele of *lrp*, because of a resemblance in the phenotypes conferred by mutation of these loci on LIV-I expression (6a). Extensive genetic, physiological, and molecular studies also suggest that expression of the LIV-I genes may be influenced by the intracellular levels of charged leucyl-tRNA and the Rho transcription termination factor (17, 18, 27–30). A Rho-dependent transcription termination mechanism regulated by the intracellular levels of charged leucyl-tRNA has been proposed to function in the 5′ untranslated leader regions (1) preceding the *livJ* and *livKHMGF* transcription units (17).

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We report here an in vivo analysis of the regulation of transcription of the *livJ*-binding protein gene. Temperature-sensitive alleles for leucyl-tRNA synthetase (EC 6.1.1.4) and the Rho transcription termination factor were introduced into a mutant strain which lacked repression control of transcription initiation of the LIV-I transport genes. Analyses of mRNA synthesis in the various mutant strains indicated that both shortages of leucyl-tRNA caused by inactivation of the temperature-sensitive leucyl-tRNA synthetase and inactivation of Rho factor are associated with increased transcription of *livJ*. Prematurely terminated transcripts, corresponding in size to the leader region of *livJ* mRNA, were detected. Intracellular accumulations of these short transcripts were preferentially reduced in strains harboring either temperature-sensitive alleles for leucyl-tRNA synthetase or Rho factor. We, therefore, suggest that expression of *livJ* involves Rho-dependent transcription termination where antitermination is associated with the intracellular availability of aminoacyl leucyl-tRNA.

MATERIALS AND METHODS

Bacterial strains, phage, and media. *E. coli* K-12 strains used in this study are described in Table 1. Preparation of LB and M-9 minimal growth media was done as described by Miller (24). M-9 minimal growth medium was supplemented with amino acids, pyridoxine, and thymine at 50 μg/ml, and glucose was used at 0.2%. The *E. coli* generalized transducing bacteriophage P1*vir* (a gift from D. Friedman) was used according to established procedures (24) for the construction of strains.

Measurements of mRNA synthesis. (i) Growth conditions. Cells were grown to exponential phase in LB medium at 30°C, recovered by centrifugation (5,000 × *g*, 5 min, 4°C), washed in M-9 minimal media, and resuspended to an optical density of 0.05 at 420 nm in M-9 minimal medium supple-

TABLE 1. *E. coli* bacterial strains used

Strain	Genotype	Source or reference
AE40	*metB*$^+$; otherwise as JC1552	Laboratory strain collection
AE52	*livR1*; otherwise as AE40	Laboratory strain collection
AE500	*leuS31*; otherwise as AE52	This study, P1(AE52 × KL231)
AE501	*rho-112 ilv-683*; otherwise as AE52	This study, P1(AE52 × CGSC6108)
GCSC6108	*thr-33 trpE9829*(Am) *tyrA15*(Am) *thyA707 ilv-683 rho-112*(Am) *argH* λ$^-$ *supF* IN(*rrnD-rrnE*)*1*	B. Bachmann, *E. coli* Genetic Stock Center
JC1552	*argG6 his-1 metB1 leu-6 trp-31 mtl-2 xyl-7 malA1 gal-6 lacY1 str-104 tonA2 tsx-1 supE44* Nal^r λ$^-$ F$^-$	27
KL231	*leuS31 thyA deoC rpsL*	B. Bachmann, *E. coli* Genetic Stock Center

mented with amino acids, pyridoxine, thymine, and glucose. Cultures were allowed to grow for at least three doublings at 30°C and resuspended to a final optical density of 0.05 at 420 nm in fresh M-9 minimal medium that had been preincubated at either 30 or 42°C. Growth proceeded for at least three doublings at each temperature to ensure balanced growth prior to sampling.

(ii) Preparation and isolation of ^{3}H-labeled RNA. Cultures were isotopically labeled according to modifications of previously described methods (21, 24, 36). Each culture was mixed with 0.5 mCi of [5,6-^{3}H]uridine (45 Ci/mmol) (Amersham) for 2 min, suspended in 1 ml of lysis buffer (0.02 M Tris-HCl [pH 7.4], 0.2 M NaCl, 0.04 M EDTA, 100 μg of carrier yeast RNA per ml, and 1% sodium dodecyl sulfate [SDS]), and heated to 100°C in boiling water. Samples were cooled to room temperature, and an equal volume of H_2O-saturated phenol was added to each sample. The mixtures were subsequently shaken for 15 min at room temperature. After centrifugation (2,500 × *g*, 5 min), the aqueous phase was removed, and the phenol extraction was repeated. Nucleic acids were precipitated from the aqueous phase by adding a 1/10 volume of 3 M sodium acetate and 2 volumes of ethanol. After centrifugation, pellets were lyophilized to dryness and stored at −20°C.

(iii) DNA-RNA hybridization. The DNA probes used were derived by restriction endonuclease digestion of the pOX15 plasmid (*bla*$^+$ *tet livJ*$^+$), a derivative of pBR322 that harbors a cloned insert containing the *livJ* structural gene and 5′ sequences. A 9-μg sample of a purified 700-bp *Pvu*II DNA fragment (see Fig. 2, probe II) was denatured and immobilized onto a GeneScreen Plus nylon membrane filter (6 by 10 cm; New England Nuclear) according to the vendor's recommendations. Small filters, with a diameter of 6.5 mm, were prepared from the large filter by using a sterile hole puncher and dried at room temperature.

Hybridizations were performed in plastic scintillation vials. Filters with DNA probes were placed in separate vials, and 1 ml of hybridization solution (0.2 M Tris-HCl [pH 8.0], 0.6 M NaCl, 0.02 M EDTA, 0.1% SDS, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 50% formamide) was added to each vial. Labeled, lyophilized RNA was resuspended in a 2× sodium citrate solution (1× sodium citrate solution is 15 mM sodium citrate plus 150 mM NaCl [pH 7.0]) and pipeted into each vial. Hybridization reaction mixtures were incubated for 48 h at 42°C with gentle agitation. The filters were washed, treated with RNase A, and counted as described by Zengel et al. (36).

Detection of terminated transcripts by analysis with S1 nuclease and RNase A. Total cellular RNA, pulse-labeled with [^{3}H]uridine, was prepared from 20-ml cultures as described above, with the following exceptions. Cultures were isotopically labeled for 2 min, 2 ml of 0.02 M sodium azide was added, and the cultures were rapidly chilled in a dry ice-ethanol bath. Cells were recovered by centrifugation (5,000 × *g*, 5 min, 4°C), and the resulting pellet was resuspended in 1 ml of lysis buffer. Isolation and extraction of labeled nucleic acids were performed as previously described, with the exception that pellets were dried at room temperature. [^{3}H]RNA was resuspended in 30 μl of hybridization buffer {40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 6.4], 1 mM EDTA [pH 8.0], 0.4 M NaCl, 80% formamide} along with unlabeled 724-bp *Pvu*II-*Pvu*II DNA fragment (see Fig. 2, probe I). The mixture was incubated at 42°C for 16 to 24 h to allow the formation of hybrids in solution. Analyses of the hybrids by using S1 nuclease and RNase A were carried out as described by Atweh et al. (5). The digested samples were precipitated in 2 volumes of ethanol; resuspended in a 15-μl solution of 80% (vol/vol) formamide, 10 mM NaOH, 1 mM EDTA, 0.1% (wt/vol) xylene cyanol, and 0.1% (wt/vol) bromophenol blue; and heated at 90°C for 2 min. Products of the S1 nuclease-RNase A digestion were analyzed by electrophoresis on 8% polyacrylamide gels containing 7 M urea. An unlabeled 123-bp ladder (Bethesda Research Laboratories) was loaded into parallel lanes of the gel as size markers. To detect the [^{3}H]RNAs, the polyacrylamide gels were fixed in 10% acetic acid, washed for 1 h with five successive changes of H_2O, soaked in 1 M sodium salicylate for 1 h, and dried (7). RNAs were visualized by autoradiography with Kodak XAR-5 film and an intensifying screen at −70°C for 3 weeks.

Distributions of nuclease-resistant [^{3}H]RNA hybrids were quantitated by cutting gel lanes into 1-cm slices. [^{3}H]RNAs were eluted from each gel slice in an elution buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, EDTA [pH 8.0], 0.1% SDS), precipitated in 2 volumes of ethanol, dried, and added to a scintillation cocktail for counting.

RESULTS

Construction of AE500 and AE501 strains. To facilitate in vivo studies of the influence of Rho factor and variations in the intracellular levels of charged leucyl-tRNA on transcription of *livJ*, two strains were constructed by introduction of temperature-sensitive alleles for Rho factor (*rho-112*) and leucyl-tRNA synthetase (*leuS31*) into strain AE52. Strain AE52 is a leucine auxotroph which lacks repression of transcription initiation of LIV-I gene expression because of a mutation in *livR*. Previous studies have shown that strains harboring a mutation in *livR* display derepressed expression of the LivJ-binding protein with increased LIV-I transport activity (18). Phage P1*vir* lysates were prepared with strain KL231 (*leuS31*) and used for transduction into strain AE52.

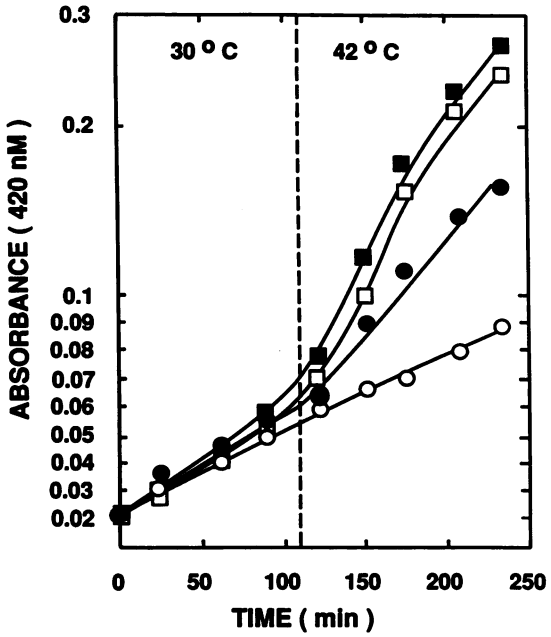

FIG. 1. Growth response of *E. coli* strains to temperature shifts. Strains were grown initially at 30°C on glucose-minimal media and, at the indicated time, shifted to growth at 42°C. Symbols: ■, AE40 (*livR*$^+$); □, AE52 (*livR1*); ●, AE500 (*livR1 leuS31*); ○, AE501 (*livR1 ilv-683 rho-112*).

A selection scheme for the *leuS31* allele was developed by screening transductants resistant to growth on 100 μg of azaleucine per ml for temperature sensitivity and retardation of growth when isoleucine and valine are included on solid media (22), yielding strain AE500. The ability of the *leuS31* allele in strain AE500 to reduce intracellular charging of leucyl-tRNA when the enzyme was inactivated by growth at a restrictive temperature was verified by an in vivo aminoacylation assay for $tRNA^{Leu}$ (15). Phage P1*vir* lysates prepared with strain CGSC6108 were used to transduce the *rho-112* allele into strain AE52. Because of the close linkage between *rho* and *ilv* (61% cotransducible [14]), the *rho-112* allele was selected by screening Ilv$^-$ transductants for temperature-sensitive growth at 42°C to yield strain AE501. Figure 1 shows that AE500 and AE501 strains demonstrate temperature-sensitive growth characteristics compared with their isogenic parental strains. The abnormally slow growth of strain AE501 was consistent with a previous report of abnormal cell growth in strains harboring the *rho-112* allele when grown on minimal-salts media (14).

Detection of in vivo [^{3}H]uridine-labeled transcripts corresponding to the *livJ* leader. The proposed structural organization of *livJ* is schematically shown in Fig. 2. The *livJ* structural gene contains a leader region of 104 nucleotides, which precedes the first AUG initiation codon. The start of translation of the *livJ* mRNA has been established by sequence analysis of the LivJ-binding protein (25). Transcription that initiates mRNA synthesis has been postulated, from in vitro transcription studies, to either continue into the structural gene or prematurely terminate after the leader transcript has been synthesized (17). *livJ* mRNAs were analyzed for evidence of prematurely terminated transcription. The standard technique of blotting unlabeled total cellular RNAs onto a membrane support (Northern [RNA] analysis) would not permit a clear distinction between primary transcripts and degradation intermediates of a full-length message, since mRNA is rapidly degraded in procaryotes. Therefore, an alternative approach in which newly synthesized RNAs were isotopically pulse-labeled in vivo was adopted.

[^{3}H]RNA species were hybridized in solution to a 724-bp *Pvu*II-*Pvu*II fragment (Fig. 2, probe I) containing sequences for the promoter, leader, and 5′ end of the *livJ* structural gene. Hybrids were digested with a combination of S1 nuclease and RNase A, and nuclease-resistant products were analyzed by gel electrophoresis and radiography. Transcript species of approximately 100 to 120 bp in size were detected among newly synthesized RNA in strain AE52, in which *livJ* expression was derepressed as a result of a mutation in *livR* (Fig. 3, lane 3). The transcript species were also apparent among newly synthesized cellular RNAs of strains AE500 and AE501 (Fig. 3, lanes 2 and 4). However, these species were absent among newly synthesized RNAs isolated from strain AE40 (Fig. 3, lane 1) harboring a wild-type *livR*$^+$ locus, in which expression of *livJ* is repressed at the concentration of leucine used in this study. In each strain harboring a *livR* mutant genotype, a 514-bp read-through transcript was apparent (Fig. 3).

***leuS31* and *rho-112* mutations alter accumulation of terminated and read-through transcripts.** Influence of *leuS31* and *rho-112* mutations on the distribution of *livJ* [^{3}H]mRNA, which was protected from nuclease cleavage, was examined. Nuclease-resistant hybrids were fractionated on a denaturing polyacrylamide gel and quantitated. Newly synthesized

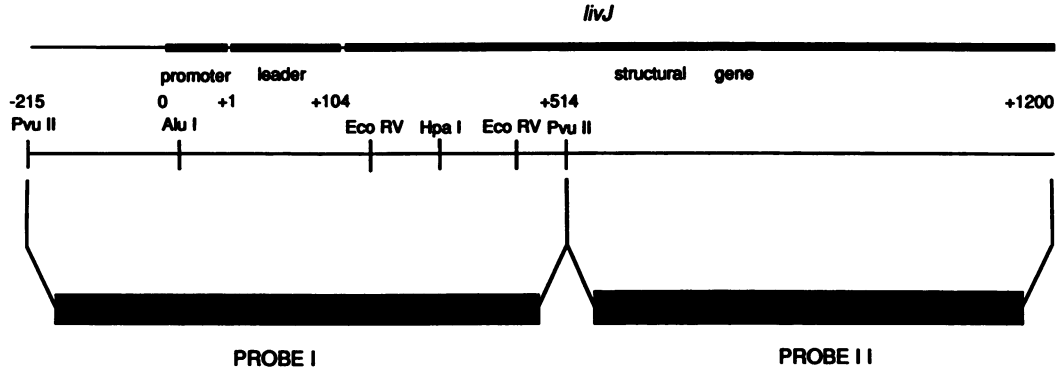

FIG. 2. Physical map of *livJ*. The top line describes the proposed organization of *livJ*. Base positions are numbered relative to the start of transcription. DNA probes used in this study are shown in relation to the restriction map of *livJ*.

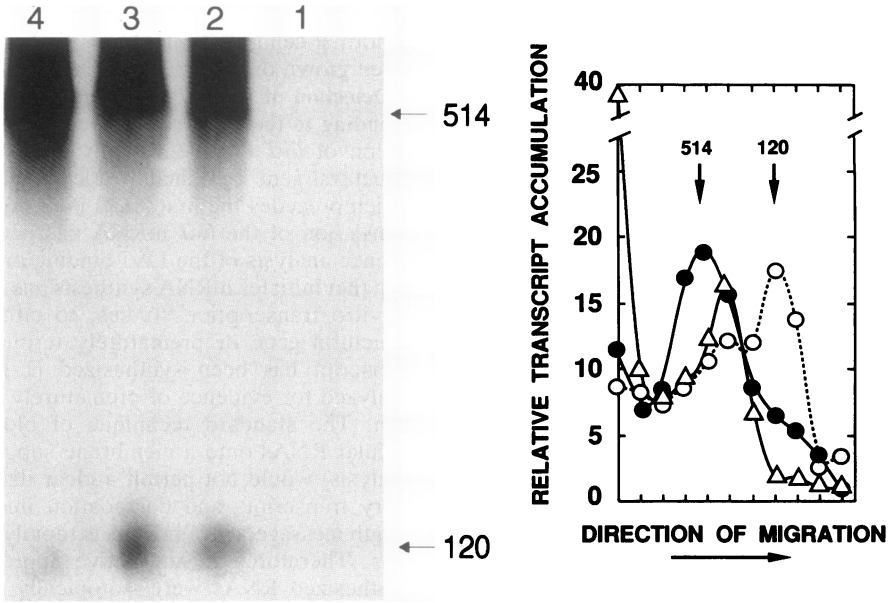

FIG. 3. In vivo detection of [^{3}H]RNAs corresponding to the *livJ* leader. Total cellular RNAs, briefly labeled with [^{3}H]uridine, were isolated from cells grown at 42°C, as described in Materials and Methods. Labeled RNAs were hybridized to a 724-bp *Pvu*II-*Pvu*II fragment (Fig. 2, probe I), and the resultant hybrids were incubated in the presence of S1 nuclease and RNase A. Nuclease-resistant hybrids were fractionated on an 8% polyacrylamide–7 M urea gel and visualized by autoradiography with a sodium salicylate fluor. Lanes: 1, AE40 (*livR*$^+$); 2, AE500 (*livR leuS31*); 3, AE52 (*livR*); 4, AE500 (*livR rho-112 ilv-683*). The distribution of [^{3}H]RNAs that were protected from nuclease cleavage by probe I were quantitated as described in Materials and Methods. Values for relative accumulation were normalized by dividing the counts per minute eluted per fraction by the total counts per minute eluted in a given lane × 100. Symbols: ○, AE52; ●, AE500; △, AE501.

transcripts protected from nuclease cleavage from strains AE500 and AE501 showed reduced accumulation of the 100- to 120-bp transcripts compared with transcripts from nuclease-resistant hybrids isolated from their isogenic parent strain (Fig. 3). In contrast, the proportion of transcripts of sizes greater than the 100- to 120-bp truncated species, representing potential read-through transcripts, was increased among the newly synthesized RNAs of strains AE500 and AE501 compared with that of their parental AE52 strain.

Effects of *leuS31* and *rho-112* alleles on synthesis of *livJ* mRNA in vivo. We compared the effect of the *leuS31* and *rho-112* alleles on transcription of *livJ* by DNA-RNA hybridization. Various concentrations of total cellular RNA derived from cells grown at either 30 or 42°C and labeled with [^{3}H]uridine were hybridized to either probe I, specific for the first 514 bases of *livJ*, or probe II, corresponding to bases 515 through 1,200 of the *livJ* structural gene (Fig. 2).

Inactivation of leucyl-tRNA synthetase by growth of strain AE500 at 42°C was associated with increased amounts of transcripts corresponding to both probes I and II (Fig. 4) compared with the levels observed during growth at the permissive temperature. In contrast, inactivation of Rho factor resulted in reduced accumulations of transcripts which hybridized to probe I and a slight increase in accumulation of transcripts which hybridized to probe II. Growth of strain AE52 at 42°C was associated with reduced accumulation of transcripts which hybridized to probe I and increased levels of transcripts corresponding to probe II compared with RNAs from cells grown at the permissive temperature (Fig. 4). The reason for the reduced accumulation of transcripts which hybridize to probe I during growth of strain AE52 at 42°C is unclear. It is known that the rates of synthesis of at least 10 aminoacyl-tRNA synthetases are

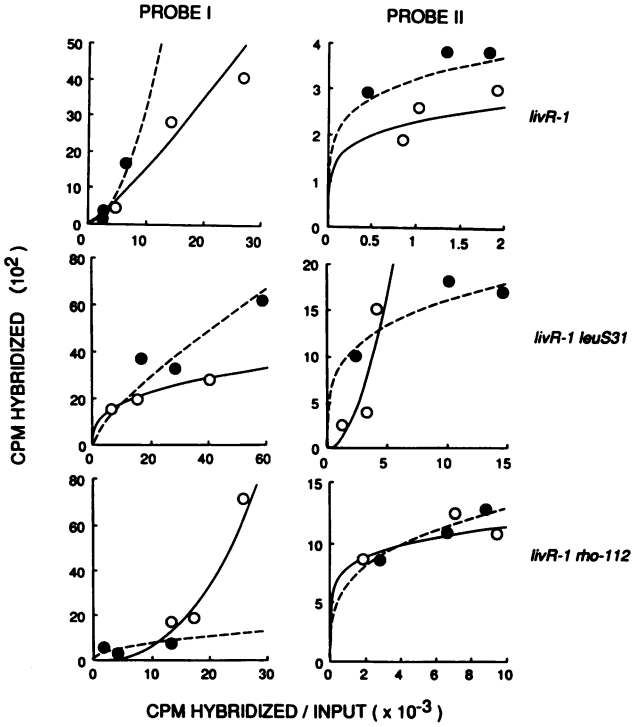

FIG. 4. Effects of inactivation of a temperature-sensitive leucyl-tRNA synthetase and Rho factor on synthesis of *livJ* mRNA. Cells were grown at either 30 (○) or 42°C (●) for at least three doublings and isotopically labeled with [^{3}H]uridine. [^{3}H]RNA was purified from the labeled cells and hybridized to filters, containing either probe I or probe II (Fig. 2), that were immobilized on membrane filters.

affected by temperature shifts (20). Thus, it is possible that a potential reduction in synthesis of leucyl-tRNA synthetase in strain AE52 during growth at high temperatures may be associated with reduced accumulation of prematurely terminated transcripts corresponding to the 5′ leader region.

DISCUSSION

Preceding the 5′ terminus of the *livJ* structural gene is a leader sequence that is 104 nucleotides in length. Involvement of the leader region in modulating the frequency of *livJ* mRNA elongation was initially suggested from physiological studies with strains harboring mutations in *rho* (18, 29, 30). Additional support for the hypothesis was provided by the demonstration that addition of purified Rho factor during in vitro transcription of *livJ* caused premature transcription termination (17).

Transcripts similar in size to the *livJ* mRNA leader sequence were detected among the total cellular RNAs of a strain containing wild-type *rho* (Fig. 3). Production of the transcript species apparently requires active transcription of *livJ*, since the RNA species were absent among total cellular RNAs derived from a strain in which *livJ* expression is repressed. Furthermore, the apparent sizes of the transcripts agree approximately with estimates for the sizes of terminated species derived from in vitro transcription of *livJ* in the presence of purified Rho factor (17). Accumulation of these species was reduced, with a concomitant increase in the appearance of higher-molecular-weight species under conditions in which the Rho factor protein was defective (Fig. 4). These results collectively lead us to conclude that in vivo Rho factor may act to terminate mRNA elongation in the leader region of *livJ* mRNA.

Previous physiological studies revealed that strains defective in either the tRNA maturation enzyme, pseudouridylate synthase, or leucyl-tRNA synthetase display increased LIV-I transport activity, implicating leucyl-tRNA as a regulatory factor (27–29). However, direct involvement of shortages in aminoacylated species for $tRNA^{Leu}$ on modulating transcription of the LIV-I genes has not been demonstrated. By manipulating the intracellular levels of aminoacyl leucyl-tRNA, through inactivation of a temperature-sensitive leucyl-tRNA synthetase, we were able to examine the influence of variations in charging of $tRNA^{Leu}$ on transcription of *livJ*. Inactivation of the temperature-sensitive leucyl-tRNA synthetase was associated with increased synthesis of *livJ* mRNA (Fig. 4). These results suggest that shortages of charged leucyl-tRNA are associated with changes in the frequency of transcription termination in the leader region of *livJ* mRNA.

From these studies, both Rho factor and shortages of charged leucyl-tRNA are believed to be components of a common regulatory mechanism, since both *rho* and *leuS* mutant alleles were associated with increased mRNA synthesis and suppression of premature transcription termination. We postulate that variations in the intracellular concentration of aminoacylated $tRNA^{Leu}$ may be associated with preemption of Rho-dependent transcription termination, since the increase in *livJ* mRNA production in strain AE500 (*leuS31 rho*$^+$) occurs in the presence of a functional wild-type *rho* allele. However, the possibility cannot be excluded that these factors may be, at least in part, unrelated and are involved in quite different functions. Indeed, recent studies have provided evidence for a direct role of leucyl-tRNA and leucyl-tRNA synthetase in the mechanism for transport of extracellular leucine (34).

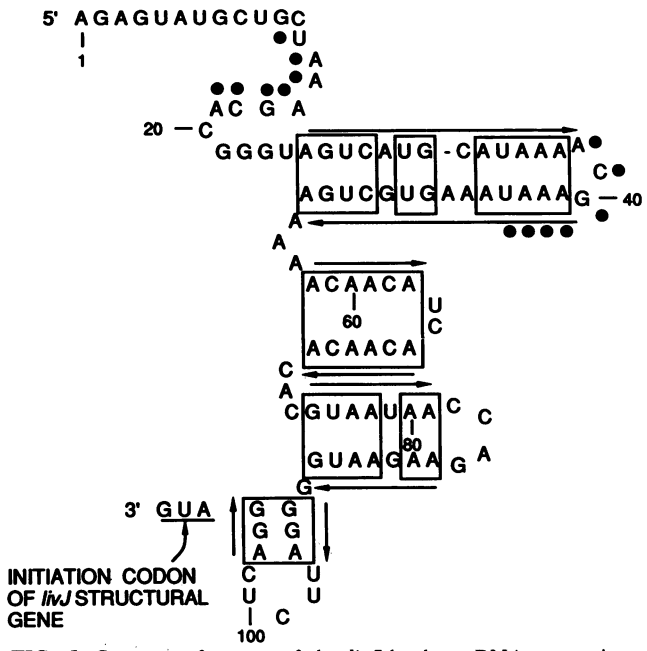

FIG. 5. Sequence features of the *livJ* leader mRNA transcript. The sequence is numbered every 20 bases from the 5′ end. Conserved, contiguous sequences having mirror image symmetry repeats are boxed, and noncontiguous mirror image repeats are indicated by closed circles. The 5′, 3′ orientation of each contiguous repeat sequence is indicated by arrows.

The mechanism for regulation of Rho-dependent transcription termination by variations in the intracellular levels of charged leucyl-tRNA is unknown. Nearly 70% of the nucleotides of the *livJ* leader mRNA are arranged in contiguous sets of repeats which display mirror image symmetry (Fig. 5). Sequences with mirror image symmetry have opposite 5′, 3′ orientations. Higgins and Ames (13) were the first to call attention to sequences having mirror image symmetry as potential recognition sites for dimeric or multimeric protein-nucleic acid interactions in single-stranded DNA and RNA. Possibly, reduced charging of $tRNA^{Leu}$ species might alter the expression or activity of a *trans*-acting regulatory factor, which may function by binding to *livJ* mRNA, preventing premature transcription termination caused by Rho factor. Evidence for antitermination control of gene expression mediated by *trans*-acting factors has been reported to regulate expression of the *bgl* (23), *sfrA* and *sfrB* (6, 10), *tna* (32, 33), and *rrn* (4) genes in *E. coli*; lambdoid bacteriophage genes (11); and the *pur* (35), *trp* (15, 31), and *sac* (8, 12) genes of *Bacillus subtilis*. A detailed mapping of *cis*-acting sites responsible for antitermination control by the intracellular levels of aminoacyl-$tRNA^{Leu}$ should clarify features of this antitermination process.

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