Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 302–306, January 1993
Genetics

Multiple control of *Escherichia coli* lysyl-tRNA synthetase expression involves a transcriptional repressor and a translational enhancer element

(downstream box/heat induction/leucine regulon/*lrp*/*lysU*)

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Communicated by Marianne Grunberg-Manago, October 5, 1992

ABSTRACT **Lysyl-tRNA synthetases [L-lysine:tRNA^{Lys} ligase (AMP-forming), EC 6.1.1.6] are synthesized from two distinct genes in *Escherichia coli*, *lysS* (constitutively) and *lysU* (inducibly), but neither the physiological significance nor the mechanism of differential regulation of these two genes is understood. We have constructed a null mutation of *lysS* that causes cold-sensitive lethality and then used this mutant to acquire and characterize several bypass mutations called *als* (*a*bandonment of *lysS*). Cold-resistant survivors were isolated either spontaneously or by transposon-mediated disruption, and all caused derepression of *lysU* transcription. One class of *als* mutations is linked to *lysU* and presumably affects the cis regulatory element. Mutations of the other class map within the *lrp* gene, which encodes the leucine-responsive regulatory protein (Lrp). A *lysU-lacZ* gene fusion study revealed that *lysU* is susceptible to thermal regulation in the absence of *lrp* and that a small mRNA region immediately downstream of the initiation codon is required for potentially high-level expression. These results suggest that *lysU* is part of the leucine regulon and is both negatively controlled by Lrp and positively regulated by a potential translational enhancer sequence. This sequence is similar to that of the "downstream box" complementary to nucleotides 1469–1483 of 16S rRNA, which can be universally found in tRNA synthetase genes of *E. coli*.**

Escherichia coli has two forms of lysyl-tRNA synthetase [LysRS; L-lysine:tRNA^{Lys} ligase (AMP-forming), EC 6.1.1.6] (1, 2), and it stands as a rare exception to the rule of one synthetase per amino acid. However, the two proteins have identical aminoacylation activities *in vitro*, so a rationale for this exception is not immediately obvious. Characterization of their structural genes, *lysS* and *lysU*, has also shown that their derived amino acid sequences are 86% identical (3–5). Nevertheless, the occurrence and stable inheritance of two LysRS genes suggest that, in spite of their apparent similarity, there might exist a functional significance for the presence of two genes that is not yet appreciated. A distinction between the possible physiological roles of *lysS* and *lysU* may be revealed by identifying differences in the mechanisms regulating the expression of the two genes.

The major species of LysRS is encoded by *lysS* located at 62 min on the chromosome (1), and the minor species is encoded by *lysU* located at 93.5 min (2). *lysS* is in the same operon as the *prfB* gene, which encodes peptide-chain-release factor 2 (3) and, like other synthetases, is expressed constitutively under all measured growth conditions (6, 7). On the other hand *lysU* is normally silent, being expressed only during growth at high temperatures or in the presence of certain metabolites such as L-alanine, L-leucine, L-leucine dipeptide, and glycyl-L-leucine (6, 8, 9). It is not presently known how metabolites induce *lysU* expression, and heat shock, the one stimulus that is generally more familiar, eludes conventional reasoning because a canonical heat-shock promoter sequence is not present in the 5′ flanking region of *lysU* (5). Genetics has also been applied to this problem with the notion that disruption of either chromosomal gene will enable a clearer investigation of individual gene activity. A strain that is null for *lysU* was created, but it exhibited no remarkable phenotype other than slightly slower growth at high temperatures (5). On the other hand, disruption of *lysS* confers cold-sensitive growth to the cells, and this cold sensitivity is suppressed by multicopy plasmids carrying *lysU* (10). Therefore, the *lysS* gene is dispensable at high temperature because of heat-inducible expression of *lysU* but is indispensable at low temperatures because of the lack of expression from either gene. Thus, the *lysS* gene is functionally replaceable by *lysU* for cell growth. This study focuses on the mechanism of *lysU* gene regulation revealed by mutational analysis of the null *lysS* strain and by gene fusion analysis. From the results reported here we argue that *lysU* is controlled as a component of the leucine regulon at the transcriptional level and offer evidence for the involvement of a potential enhancer element at the translational level.

MATERIALS AND METHODS

Bacterial and Phage Strains. Bacterial strains (*E. coli* K-12) used, except for the β-galactosidase assay, were W3110 (F$^-$ prototroph) and its derivatives: KK694 (*lysS1*::Cm^r*zgc-21*::Tn*10*) (10), KK696 (*lysS1*::Cm^r) (10), RM377 (*lysS1*::Cm^r*lrp111*::ΔTn*10*Kan), RM378 (*lysS1*::Cm^r*lrp112*::ΔTn*10*Kan), RM379 (*lysS1*::Cm^r*lrp113*::ΔTn*10*Kan), RM398 (*lrp112*::ΔTn*10*Kan), RM451 (*lysS1*::Cm^r*lrp114*), RM452 (*lysS1*::Cm^r*lrp115*), RM453 (*lysS1*::Cm^r*lrp116*), RM454 (*lysS1*::Cm^r*lrp117*), and RM455 (*lysS1*::Cm^r*lrp118*) where Cm = chloramphenicol, Kan = kanamycin; and Cm^r = Cm-resistant. Strain MC4100 (F$^-$ *araD* Δ(*argF-lac*)*U169 rpsL relA flbB deoC ptsF rbsR*) (11) was used for the β-galactosidase assay. Strains RM377 through RM455 were isolated in this study as cold-resistant revertants from KK694 or KK696. P1*vir* phage was used for transduction mapping. λNK1105 ("λ hop") carrying a transposable ΔTn*10*Kan element was used for disruption mutagenesis (12). Kohara's phage λ clone 213 of the ordered genomic library (13) was used as a source of DNA for the wild-type *lrp* gene. λLRP phage carrying the *lrp* gene was constructed by *in vivo* transfer of the *lrp* segment from the pfRP12 plasmid to λRZ5 phage (14) by *recA*-mediated recombination as described (15). λfRM460 and λfRM461 phages carrying *lysU-lacZ* gene fusions were constructed by the same

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Abbreviations: Cm, chloramphenicol; Kan, kanamycin; Cm^r and Kan^r, Cm- and Kan-resistant; LysRS, lysyl-tRNA synthetase.

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in vivo recombination method using plasmids pRP77 and pKK515, respectively.

Selection of the *als* Mutants. The *lysS1* mutant grows normally at 42°C but fails to grow at ≤30°C. The cold-sensitive growth phenotype of the *lysS1* mutant enabled us to isolate second-site mutations that allowed constitutive expression of the *lysU* gene in cold-resistant revertants. Forty-three such mutants were selected and characterized in this study. These suppressor mutations were designated *als* for *a*bandonment of *lysS*.

Plasmids. Plasmid pLRP was constructed by recloning the 2.5-kilobase (kb) *Eco*RI fragment containing the *lrp* gene from the Kohara's λ clone 213 into the same site of pACYC184. This *Eco*RI fragment was also cloned at the same site of pMC1403 (16), generating pfRP12. The Kan-resistant (Kan^r) fragment generated with *Bam*HI from pUC-4K (17) was inserted at the *Bgl* II site of pLRP, giving rise to plasmid pLRP(−)Kan (Fig. 1*C*). Plasmid pIT134 carries a Cm^r cassette between *Bgl* II and *Bam*HI sites of plasmid pEG5005 (18), which contains a mini-Mu replicon (*c*ts62 transposition–replication gene A^+B^+) and a ColE1 replicon.

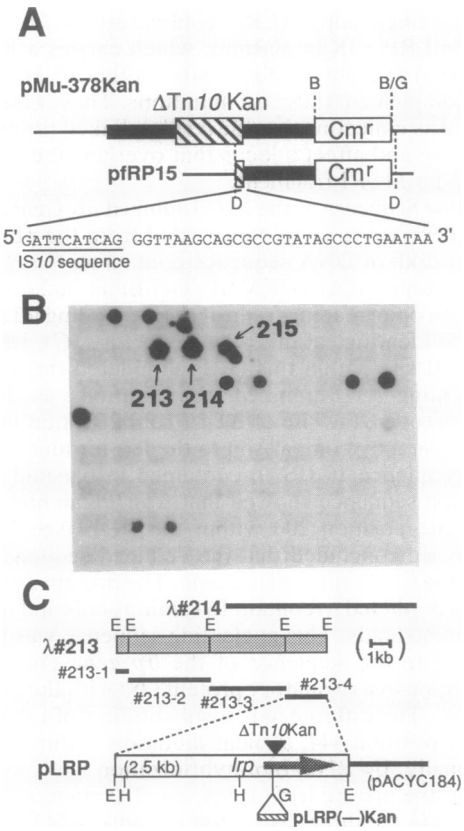

FIG. 1. Cloning and structure of the DNA fragment carrying the *lysU*-unlinked *als* (i.e., *lrp*) gene. Only relevant restriction sites are included: B, *Bam*HI; D, *Hin*dIII; E, *Eco*RI; G, *Bgl* II; H, *Hpa* I. (*A*) Structure of the mutant DNA (*lrp112*::ΔTn*10*Kan) cloned in the plasmids. Bold bars indicate the bacterial DNA, and thin bars indicate plasmid DNAs. The open and the hatched boxes represent insertions of the Cm^r gene and the ΔTn*10*Kan transposon, respectively. Part of the sequence containing the *lrp112*::ΔTn*10*Kan junction is shown. (*B*) Screening of *lysU*-unlinked *als* by DNA blot-hybridization analysis. The *ca.* 4.0-kb *Hin*dIII insert was isolated from the pfRP15 plasmid, labeled with [α-^{32}P]dCTP by random priming, and used for hybridizing a gene-mapping-membrane containing the Kohara's mini-set genomic DNAs. Positive spots other than the clones 213, 214, and 215 are those that reacted to traces of transposons in the genome. (*C*) Chromosomal structure of DNAs cloned in the Kohara phages and plasmids. Sites of Kan^r and ΔTn*10*Kan insertions are noted by triangles. The arrow represents the location and orientation of the *lrp* gene.

Plasmids pRP77, pRP92, pRP101 through pRP106, and pKK515 contain several *lysU-lacZ* gene fusions and were constructed by ligating amplified *lysU* DNAs to pMC1403. The 4.0-, 0.54- to 0.56-, and 0.31-kb DNAs were amplified from the plasmid DNA pFN120 (5) by polymerase chain reaction (PCR) (19) using nine sets of primers: K84 (CCGGATCCTATGAAAACATGCGTAT)–K83 (CCGGATCCTGATCTTCTGCGTCGTT), K98 (CCGGAATTCAGCTTGTTTATCGATC)–K44 (GGGATCCATTTTGGTTCCTCTAAATC), K98–K101 (GGTGATCAGACATTTTGGTTCCTCTAA), K98–K102 (GGAGATCTTCAGACATTTTGGTTCCTC), K98–K103 (GGAGATCTTGTTCAGACATTTTGGTTC), K98–K104 (GGAGATCTTCTTGTTCAGACATTTTGG), K98–K105 (GGAGATCTGTTTCTTGTTCAGACATTT), K98–K106 (GGAGATCTCCCCGTGTTTCTTGTTCAG), and K43 (GGAATTCATCGTCGTATTGGCCTTTG)–K44, respectively. The termini of amplified DNAs were tagged with *Bam*HI sequences with PCR primers, and these DNAs were cloned in frame to *lacZ* into the same restriction site of pMC1403. Gene fusions resulted at *lysU* codon positions 432, 1 through 6, 8, and 1 in pRP77, pRP92, pRP101 through pRP106, and pKK515, respectively, and were confirmed by nucleotide sequence analysis (data not shown).

***In Vivo* Cloning of *lrp112*::ΔTn*10*Kan.** *E. coli* strain RM398 (*lrp112*::ΔTn*10*Kan) lysogenic for Mu cts62 phage (18) was transformed with the pIT134 plasmid by selecting for Cm^r at 30°C. The transformant cells were grown in LB medium (20) at 30°C, and phage lysates were prepared by shifting growth temperatures to 42°C. During the heat induction, transposition of the mini-Mu replicon can occur on both sides of the *lrp112*::ΔTn*10*Kan insert to form a structure that can be encapsidated by the efficient Mu headful packaging mechanism (21). The resulting lysates were used to infect Mu-lysogenic W3110 cells, where recombination can occur between the Mu sequences to generate the mini-Mu plasmid. Kan^r colonies were selected at 30°C that contain the pMu-378Kan plasmid carrying the *lrp112*::ΔTn*10*Kan and flanking sequences (Fig. 1*A*). The *ca.* 4-kb *Hin*dIII fragment containing the right-hand junction of ΔTn*10*Kan, *lrp*, and Cm^r was recloned to the same site of pUC119, giving rise to pfRP15, which was analyzed in this study (see Fig. 1*A*).

RNA Blot-Hybridization Analysis. RNAs were isolated from *E. coli* cells by the method of Aiba *et al.* (22), subjected to agarose-gel electrophoresis in the presence of formaldehyde, and blotted onto Hybond-N+ filters (Amersham) as described by Sambrook *et al.* (23). The *lysU*-specific radioactive DNA probe was synthesized by PCR using *lysU*-specific primers (GGATCGATGGTTATTTATTAGTGAT and GAGGATCCTCAAAACTGGATTTGCG), [α-^{32}P]dCTP, and AmpliTaq DNA polymerase (Cetus). The *lpp*-specific DNA probe was prepared from the pKEN125 plasmid (24) by digesting with *Xba* I and *Mlu* I and labeled with [α-^{32}P]dCTP by the random priming method (23). The filters were hybridized, washed, and analyzed by BioImage analyzing system 2000 (Fuji Film, Tokyo) to quantify the intensity of radioactive bands.

Other Methods. Double-stranded plasmid DNAs were sequenced by the dideoxynucleotide chain-termination method (25) using specific primers synthesized from the *lrp* and ΔTn*10*Kan sequences (26). The DNA blot filter containing the Kohara mini-set genomic library is composed of 476 phage clones (supplied by Takara Shuzo, Tokyo).

RESULTS

Isolation and General Characterization of *als* Mutations. Forty spontaneous survivors were selected at 23°C from the strains KK694 (*lysS1 zgc-21*::Tn*10*) and KK696 (*lysS1*). By P1 phage transduction, these cold-resistant suppressor mu-

tations (called *als*) were then classified into two groups: either linked or unlinked to *lysU*. Six mutations were ≥90% cotransducible with the *lysU*-linked transposon markers, *zjd-2231*::Tn*10* (27) or *zjd-3182*::Tn*10*Kan (27). The remaining 34 *als* mutations were tested by P1 phage transduction, and all mapped at 20 min on the *E. coli* chromosome (see below).

The *lysU*-unlinked *als* mutations were characterized for their effect on *lysU* expression by RNA blot-hybridization analysis. RNAs were extracted from wild-type and mutant cells (RM451 through RM455) and hybridized to DNA probes specific for *lysU* and *lpp* (internal control) after electrophoresis (Fig. 2 *A* and *B*). The level of lysU mRNA, normalized against lpp mRNA increased 3- to 30-fold in the *als* mutants compared with wild type. Under these conditions, the lpp mRNA level did not change appreciably. A plausible explanation for increased lysU mRNA caused by the unlinked *als* mutants is that they nullify the putative repressor gene of *lysU*.

Based on the above assumption, we employed random disruption mutagenesis by using "λ hop" carrying ΔTn*10*Kan transposon. KK696 (*lysS1*) cells were infected with λNK1105, and three cold-resistant colonies (RM377 through RM379) were selected at 23°C. P1 phages grown on these survivors successfully cotransduced into strain KK696 the capacity for cold-resistant growth along with resistance to Kan. These markers did not segregate during crosses (200 of 200), suggesting that the ΔTn*10*Kan transposon insertions are solely responsible for suppression of cold-sensitive growth in *lysS1*. Furthermore, none of these Kan^r insertions cotransduced with *zjd-2231*::Tn*10*, indicating that they are not linked to *lysU*, just like the spontaneous mutations isolated previously. The RNA blot-hybridization analysis showed an increase in the lysU mRNA level of *ca.* 60- to 80-fold (Fig. 2 *C* and *D*). This derepression level did not change when the *lysS1* allele was restored to wild type (data not shown).

***In Vivo* Cloning and Detailed Mapping of the *als* Mutation.** The ΔTn*10*Kan insertion mutation in RM378 was cloned *in vivo* by using the mini-Mu plasmid system. The resulting plasmid pMu-378Kan carries 7-kb of genomic DNA including the ΔTn*10*Kan insertion (Fig. 1*A*). Its derivative pfRP15 DNA was radiolabeled with [α-^{32}P]dCTP and used to hybridize the Kohara miniset genomic filter for physical mapping (Fig. 1*B*). Because *E. coli* DNA sustains traces of transposable elements at several chromosomal loci, those background signals were assigned from hybridization of the same filter to the ^{32}P-labeled ΔTn*10*Kan control DNA. The net genomic DNA carried on pfRP15 hybridized strongly to clones 213 and 214 and less strongly to clone 215. The *E. coli* DNAs in these clones overlap each other and are located at 20 min on the chromosome (Fig. 1*C*). Consistent with the physical mapping data, the Kan^r marker in RM378 was cotransducible by P1 phage with the *zbj-1230*::Tn*10* transposon located at 20 min (27) at a frequency of 60%. Likewise, the other two Kan^r-tagged mutations were capable of suppressing cold-sensitive growth in *lysS1* and were linked by P1 transduction to *zbj-1230*::Tn*10* at the same frequency (data not shown). These results suggest that *lysU*-unlinked *als* mutations are localized at a single chromosomal locus at 20 min.

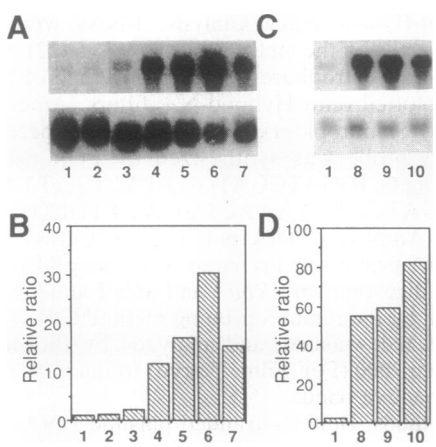

FIG. 2. RNA blot-hybridization analysis of *lysU* transcripts in the *lrp* mutants. Bulk RNA (≈3 μg) of each transcript was subjected to electrophoresis, blotted, and hybridized to the ^{32}P-labeled *lysU* and *lpp* probes. Filters were washed and analyzed by BioImage analyzing system 2000. (*A* and *C*) Autoradioimage. (*B* and *D*) Relative ratio of the lysU mRNA level to that of lpp mRNA. The intensity of lysU and lpp mRNA bands was quantified, and the ratio (lysU/lpp) was presented as a relative value to that of the wild-type (W3110) strain. Samples: 1, W3110 (*lrp*$^+$); 2, KK696 (*lrp*$^+$); 3, RM451 (*lrp114*); 4, RM452 (*lrp115*); 5, RM453 (*lrp116*); 6, RM454 (*lrp117*); 7, RM455 (*lrp118*); 8, RM377 (*lrp111*); 9, RM378 (*lrp112*); 10, RM379 (*lrp113*). Note that the level of lpp mRNA did not change appreciably.

The genomic DNA in Kohara's clone 213 was digested with *Eco*RI, and all four fragments were separately subcloned to pACYC184 (see Fig. 1*C*). The 2.5-kb fragment complemented all *lysU*-unlinked *als* mutations by both multicopy (pLRP) and single-copy (λLRP) configurations. On the other hand, the pLRP(−)Kan plasmid, which carries a Kan^r cassette inserted at a unique *Bgl* II site on the 2.5-kb fragment failed to complement these *als* mutations. These results were interpreted as indicating that the *lysU*-unlinked *als* mutations are recessive and affect a locus that overlaps the *Bgl* II site on the 2.5-kb *Eco*RI fragment.

Nucleotide Sequence of the *lysU*-Unlinked *als* Gene. Several subclone derivatives were constructed from the pLRP plasmid, and 1.2 kb of DNA sequence containing the *Bgl* II site was determined (DDBJ/EMBL/GenBank accession no. D11105). An open reading frame (ORF) encoding a 18,875-Da protein was identified at this region (see Fig. 1*C*). Disruption at the *Bgl* II site within this ORF eliminated the ability for complementing *lysU*-unlinked *als* mutations as described above. Moreover, the site of ΔTn*10*Kan insertion in RM378 was determined by comparison to the left terminal repeat of insertion sequence 10 (IS*10*) contained in plasmid pfRP15. The junction sequence showed that ΔTn*10*Kan had inserted at nucleotide position 200 within the ORF (see Fig. 1*A*). Therefore, we concluded that the deduced sequence corresponds to the *lysU*-unlinked *als* gene. The protein encoded by the ORF is predicted to contain 164 amino acids and to be rich in basic amino acids. This nucleotide sequence was found to be identical to the sequence of the *lrp* gene encoding the leucine-responsive regulatory protein (28) with the exception of one base. The difference is a substitution of cytosine for thymine at position 444, a silent mutation within the valine codon. Finally, by RNA blot-hybridization analysis we observed that the mature lrp mRNA, *ca.* 750 nucleotides long, disappeared in the three disruption mutants, RM377 through RM379 (data not shown). Therefore, in addition to its previously known roles, the Lrp protein also functions as a negative regulator of the *lysU* gene. Thus, the *lysU*-unlinked *als* mutations were named for *lrp* alleles.

Role of Lrp in *lysU* Expression. The *lrp* gene encodes a bifunctional transcription factor that activates or represses several genes involved in branched-chain amino acid metabolism (28–32). From this fact it was predicted that transcriptional control of the *lysU* gene by Lrp should be affected by L-leucine. In support of this prediction, the lysU mRNA level increased 5-fold by addition of L-leucine in the wild-type cells (data not shown). This is consistent with the previous observation that the heat-inducible form of LysRS protein is synthesized upon leucine addition (8). On the other hand, the lrp mRNA level itself was not affected by addition of L-leucine, suggesting that the derepression of *lysU* commences in some fashion other than by altering *lrp* transcription (data not

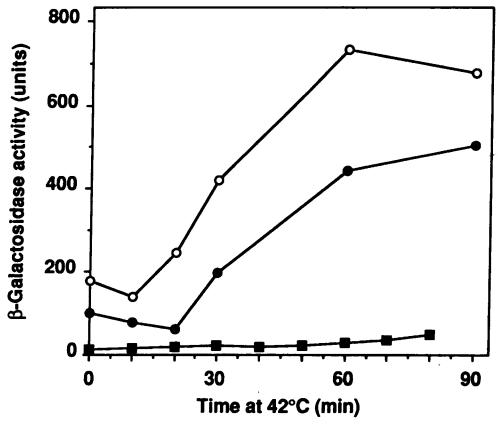

FIG. 3. Expression of *lysU-lacZ* gene fusions upon temperature shift. Isogenic *lrp*$^+$ (MC4100) and *lrp* null strains lysogenic for λ phages carrying *lysU-lacZ* gene fusions were grown in LB medium at 30°C and shifted to 42°C at time 0. Samples were taken at the times indicated and assayed for β-galactosidase activities as described (20). The values were expressed as Miller units (20). ●, MC4100 (λfRM460); ○, MC4100 *lrp112* (λfRM460); ■, MC4100 (λfRM461). The null *lrp112* mutation was transduced to the MC4100 (λfRM460) lysogen by P1 phage transduction. Partial inactivation of Lrp protein occurred in LB medium, leading to derepression of *lysU*.

shown). The cold sensitivity of the *lysS1* mutant was not suppressed by addition of L-leucine, even though this causes a 5-fold induction of lysU mRNA (data not shown).

Expression of a *lysU-lacZ* gene (protein) fusion carried on a λ prophage was examined in MC4100 (*lrp*$^+$ Δ*lac*) and its null *lrp112* cells to investigate whether heat induction of *lysU* is mediated by Lrp or not. The gene fusion (λfRM460) containing a promoter, Shine–Dalgarno sequence, and the first 1296 nucleotides of coding sequence of *lysU* exhibited a marked induction upon shift from 30°C to 42°C both in the presence and absence of *lrp* (Fig. 3). Therefore, the heat inducibility of *lysU* may not be mediated by the Lrp protein. Consistent with previous observations regarding leucine induction, the level of lrp mRNA did not change appreciably as a function of growth temperatures (data not shown).

mRNA Region Required for High-Level Expression. Contrary to the fusion on λfRM460 created at the 432nd codon of *lysU*, the gene fusion on λfRM461 at the first methionine codon did not exhibit high-level expression at any growth condition nor did it respond appreciably to temperature shift (Fig. 3). For reasoning the defect in *lysU* expression, 3′ deletions (in-frame fusions) were constructed by amplifying DNAs by PCR with different primers and subsequent cloning into plasmid pMC1403. Deletions extending up to codon 4

		matches	β-galactosidase activity (units)
16S rRNA	3′A GUA CUU AGU GUU UC 5′		
pRP92	AUG GAU CCC GUC GUU UU A CAA CGU CGU GAC UGG	2(3)/15	476
pRP101	AUG UCU GAU CCC GUC GU U UUA CAA CGU CGU GAC	4(6)/15	1476
pRP102	AUG UCU GAA GAU CCC GU C GUU UUA CAA CGU CGU	5(7)/15	1862
pRP103	AUG UCU GAA CAA GAU CC C GUC GUU UUA CAA CGU	6(7)/15	2332
pRP104	AUG UCU GAA CAA GAA GA U CCC GUC GUU UUA CAA	7(9)/15	2286
pRP105	AUG UCU GAA CAA GAA AC A GAU CCC GUC GUU UUA	8(9)/15	2618
pRP106	AUG UCU GAA CAA GAA AC A CGG GGA GAU CCC GUC	8(9)/15	2397

FIG. 4. Structures and expression of 3′ deletions. MC4100 cells carrying plasmids pRP92 and pRP101 through pRP106 were grown in LB medium containing 50 μg of ampicillin per ml at 42°C and were assayed for β-galactosidase activities as in Fig. 3. Sequences from the *lysU* start codon to *lacZ* in gene fusions carried on plasmids are presented. Nucleotides of *lysU* are underlined, and nucleotides 1469–1483 of the complement of the downstream box of *E. coli* 16S rRNA are shown at the top. The complementary nucleotides allowing G·U pairs are indicated by dots. Numbers of A·U and G·C pairs are indicated, and those in parentheses show matches allowing G·U pairs.

(pRP103) failed to alter expression; however, further deletions toward the start codon (pRP102, pRP101, and pRP92) reduced expression in proportion to the length of the deletion (Fig. 4). Therefore, we conclude that an internal coding region flanked by the first four codons is required for high-level expression. In agreement with this result, one sequence of 15 nucleotides similar to that required for enhanced expression of T7 phage gene 0.3 ("downstream box," complementary to nucleotides 1469–1483 of 16S rRNA; ref. 33) is found at this region (Fig. 4). We predict that the downstream box of *lysU* acts as a positive regulatory element in translation.

DISCUSSION

Regulation of the *lysU* Gene. Specifically induced expression of *lysU* has been a long-standing puzzle in *E. coli*. This gene would seem to be redundant because of the constitutively expressed *lysS* gene encoding LysRS. Nevertheless, *lysU* exhibits strict regulation, being normally silent except when induced by high temperature or certain nutrients and metabolites. Although the biological role of *lysU* expression remains a riddle, we have discovered a significant control mechanism of this gene—namely, that the leucine-responsive regulatory protein, Lrp, which controls the leucine regulon, also functions as the primary effector of *lysU* gene expression. Therefore *lysU* is normally repressed by the Lrp protein and is derepressed upon addition of L-leucine. These results are consistent with the recent report by Lin *et al.* (34). It is noteworthy that all 34 of the independent *als* mutations not linked to *lysU* reside in the same locus as revealed in this study: the *lrp* gene. These results suggest that Lrp may be a major control element of *lysU* expression acting in trans. On the other hand, *lysU*-linked *als* mutations situated upstream of the initiation site of the *lysU* coding sequence seem to knock out the operator site responsive to Lrp or create a new promoter in *lysU* (10).

The physiological meanings of the leucine regulon are not fully understood. In the *ilvIH* operon, there is a clear functional relationship to L-leucine, but in most other cases the meanings are not as certain. For example, it has been suggested that leucine acts as a general regulatory molecule, serving to alert cells that it and probably other amino acids are available in the medium (35). However, this does not easily account for induction of *lysU* and not *lysS* by L-leucine, although no functional difference has been found in the two LysRS enzymes synthesized from *lysU* and *lysS*. Obviously we need to explore further the function of the *lysU*-encoded synthetase, distinctively from the *lysS*-encoded synthetase, which might somehow relate to utilization of several amino acids including leucine and peptides supplied exogenously in the medium.

The heat-shock regulation of *lysU* is not yet understood. This study shows that Lrp-mediated regulation of *lysU* by metabolites is distinct from that by heat shock since the *lysU-lacZ* gene fusion carried on λfRM460 retained thermal inducibility in the absence of *lrp*. Neidhardt *et al.* have reported that thermoregulation of *lysU* depends on the heat-shock transcription factor σ^{32} (36). However, we argue that heat-shock expression of *lysU* may occur at least in part at the translational level by the following reasoning. First, a single monocistronic transcript of *lysU* (i.e., 1.6 kb long) is initiated from a σ^{70} promoter located *ca.* 90–120 base pairs upstream of the start codon (10, 37): a σ^{32} promoter sequence has not been found (5). Second, thermoshift increases lysU mRNA levels <2-fold (K.I., unpublished data), which is unable to account for the 10-fold increase in LysRS proteins produced by heat shock (5, 6, 38). Third, the deletion of a downstream box in *lysU* diminished both basal expression and high-level expression upon temperature shift. Although it cannot be excluded at present that the downstream box is required

3'AGUACUUAGUGUUUC5' 16S rRNA		matches
au gUcUGAAcaAgAAAc	*lysU*	8(9)/15
au gUcUGAAcaACAcGc	*lysS*	7(9)/15
aug aCucaAGUCGCGAAG	*metG*	8(11)/15
auggaaaagac aUAUaAccCACAAGa	*valS*	8(10)/15
au gaGUGAGgCAgAAGc	*glnS*	7(10)/15
a UgAguGAcUAUAAAu	*ileS*	7(10)/15
aUGUcGAUCcCAAuc	*serS*	7(10)/15
augacuaagc cCAUcGuUUuUAGuG	*trpS*	6(10)/15
a UgAcuAAgCcCAucG	*trpS*	8(8)/15
augaaauu caGUGAAcUGUGGuu	*pheT*	4(10)/15
augaaaa UCAaaAcUCGCuucG	*gltX*	8(9)/15
au gCAaGAGcaAUAccG	*leuS*	7(9)/15
aug cUAaaAAUCuUcAAu	*cysS*	7(9)/15
augccug UUAUaAcUCuUccuG	*thrS*	7(9)/15
augagc aaGaGcAcCGCuGAG	*alaS*	6(9)/15
augcg UacaGAAUauUGuGG	*aspS*	6(9)/15
aUGaGGAaaACcAuG	*pheS*	6(9)/15
augcaa aaGUuuGaUACcAGG	*glyS*α	5(9)/15
gug gCAaaAAaCAUucAa	*hisS*	7(8)/15
aUGgcAAgCAguAAc	*tyrS*	6(8)/15
augcaagagaaa aCuUuucUgGUGGAa	*glyS*β	4(8)/15
g UgAauAuUCAgGcuc	*argS*	6(7)/15
a UgcguAcUaGCcAAu	*proS*	6(7)/15

FIG. 5. Potential downstream boxes in tRNA synthetase genes of *E. coli*. Nucleotides 1469–1483 of the complement of the downstream box of 16S rRNA are presented at the top. The complementary nucleotides allowing G·U pairs near the start codon (underlined) are given in capital letters. Numbers in parentheses show matches allowing G·U pairs. The sequences of tRNA synthetase genes were taken from the EMBL database. Two potential downstream boxes are shown for *trpS*.

simply for high-level expression and not for thermoregulation, there is a clear example that this element plays a critical role in heat-shock regulation of rpoH mRNA translation. Nagai *et al.* have proposed that an interplay between a downstream box and a negative cis element involving secondary structure formation is important in regulating translation initiation and that transient disruption of secondary structure in response to a change in temperature represents a primary step of the heat-shock response (39). Further study on the mechanism of *lysU* translational control in *E. coli* might provide useful information on a physiological significance of *lysU* and on thermoregulation that is found ubiquitously in all organisms.

Downstream Boxes Conserved in tRNA Synthetase Genes. The purine-rich Shine–Dalgarno sequence is of prime importance for correct initiation of translation; nevertheless, after the analysis of highly expressed proteins, other potential base-pairing interactions between mRNA and 16S rRNA have been proposed to explain high translational efficiencies (40). An interplay between nucleotides 1469–1483 of 16S rRNA and the sequence downstream of the initiation codon was first found in T7 phage gene *0.3* (33) and then in the glutaminyl-tRNA synthetase gene (*glnS*) (41) and *rpoH* of *E. coli* (39). This consensus sequence has been localized in other highly expressed genes such as ribosomal protein and elongation factor genes (33). Therefore, we checked all other tRNA synthetase genes of *E. coli* than *glnS* and *lysU* for the downstream box from two criteria. First, the functional downstream box needs to be in close vicinity to the initiation codon since those found in *T7* gene *0.3*, *E. coli rpoH*, *glnS*, and *lysU* (this work) are located at nucleotide positions 14, 6, 3, and 3, respectively. Second, based on the data of the serial deletion analysis shown in Fig. 4, the minimum number of A·U and G·C matches needs to be 6 of 15 or, alternatively, 7 of 15 if G·U pairs are allowed. According to these criteria, potential downstream boxes are well conserved in all tRNA synthetase genes as presented in Fig. 5. Therefore, we propose that the downstream box is used as a general enhancer element for tRNA synthetase expression in *E. coli*.

We thank T. Inada, S. Mizuno, and R. L. Clark for plasmids used; B. S. Powell for critique and editing of the manuscript; and D. L. Court for critical reading of the manuscript. The Kohara's mini-set DNA filter was supplied by a grant-in-aid project on *E. coli* genome sequencing directed by T. Yura. This work was supported in part by grants from The Ministry of Education, Science and Culture, Japan.

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