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The *lrp* Gene Product Regulates Expression of *lysU* in *Escherichia coli* K-12

RONGTUAN LIN,[1] BRIAN ERNSTING,[2] I. N. HIRSHFIELD,[3] ROWENA G. MATTHEWS,[2] FREDERICK C. NEIDHARDT,[4] R. L. CLARK,[4] AND E. B. NEWMAN[1]*

Biology Department, Concordia University, 1455 de Maisonneuve W., Montreal, Quebec H3G 1M8, Canada[1]; Department of Biological Chemistry, The University of Michigan, Ann Arbor, Michigan 48109-0606[2]; Department of Microbiology and Immunology, The University of Michigan, Ann Arbor, Michigan 48109-0620[4]; and Department of Biological Sciences, St. John's University, Jamaica, New York 11439[3]

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In *Escherichia coli* K-12, expression of the *lysU* gene is regulated by the *lrp* gene product, as indicated by an increase in the level of lysyl-tRNA synthetase activity and LysU protein in an *lrp* mutant. Comparison of the patterns of protein expression visualized by two-dimensional gel electrophoresis indicated that LysU is present at higher levels in an *lrp* strain than in its isogenic *lrp*$^+$ parent. The purified *lrp* gene product was shown to bind to sites upstream of the *lysU* gene and to protect several sites against DNase I digestion. A region extending over 100 nucleotides, between 60 and 160 nucleotides upstream from the start of the *lysU* coding sequence, showed altered sensitivity to DNase I digestion in the presence of the Lrp protein. The extent of protected DNA suggests a complex interaction of Lrp protein and upstream *lysU* DNA.

The presence of L-leucine in the growth medium affects the expression of many gene products, including both enzymes involved in leucine biosynthesis and transport and many that are not directly involved in leucine metabolism (9, 14, 17, 22). Cells grown in minimal medium with leucine show increased levels of serine deaminase (8, 15, 21), threonine dehydrogenase (12), and one of the L-serine transport systems (5). Such cells also showed decreased levels of acetohydroxyacid synthetase III (19), the triornithine transport system, and the first enzyme of L-serine biosynthesis, phosphoglycerate dehydrogenase (9).

Regulation by leucine of some of the genes that code for these enzymes has recently been shown to involve the *lrp* gene product (9, 16). A mutant carrying an insert in the *lrp* gene, formerly known as *rbl*, *oppI*, and *ihb*, showed increased synthesis of L-serine deaminase and threonine dehydrogenase and decreased synthesis of the *ilvIH* and *serA* gene products (9, 16). The *lrp* gene specifies a DNA-binding protein that uses L-leucine as a coeffector (16). The group of genes regulated by leucine and the Lrp protein has been named the leucine regulon (9).

Lysyl-tRNA synthetase (LRS) isozyme II, the *lysU* gene product, was shown to be induced in a *metK* strain, RG62 (6). However, the overexpression of LysU protein was not corrected when strain RG62 was transformed with a plasmid containing the *metK* coding sequence (10, 11). Subsequent experiments showed that strain RG62 contains both *lrp* and *metK* mutations (9). The experiments described in this paper were intended to determine which of the two genes, *lrp* or *metK*, is responsible for regulation of *lysU*. We show here that *lysU* is regulated by *lrp* and that the Lrp protein binds to sites upstream of the *lysU* coding sequence.

* Corresponding author.

MATERIALS AND METHODS

Cultures. The strains (all derivatives of *Escherichia coli* K-12) and the plasmids used in this study are described in Table 1.

LRS assay. Cells were grown as described previously (7) in minimal medium A with 0.36% glucose at 37°C to an optical density of 0.2 at 490 nm (log phase) and then harvested and washed in a buffer consisting of 0.1 M Tris (pH 7.3) and 0.01 M $MgCl_2$. Cells resuspended in the same buffer were sonicated, and LRS was assayed as described previously (7), except that the concentrations of ATP and L-lysine used here were 2×10^{-3} and 1×10^{-5} M, respectively.

L-Serine deaminase. Assays for L-serine deaminase were carried out with toluene-treated whole cells grown in glucose-minimal medium as previously described (13).

Two-dimensional gel electrophoresis. Samples (2 ml) of cultures of exponentially growing cells (A_{420}, 0.3) were removed during exponential growth at 37°C and transferred to preheated vials containing L-[^{35}S]methionine (63 μCi; 1,056 Ci/mmol) and labeled for 5 min. Unlabeled methionine (17 μmol) was added for a 3-min chase. Extracts were prepared by sonication and processed for resolution on two-dimensional polyacrylamide gels (14) as previously described (3). The radioactivity incorporated into protein was determined by trichloroacetic acid precipitation of a small aliquot of the sample and collection of the precipitate on a glass fiber filter. The sample volume applied to the gel was adjusted so that it contained approximately 10^6 dpm. After electrophoretic analysis, gels were stained with Coomassie blue, dried, and exposed to Kodak XAR film for 2 to 7 days at −25°C.

Isolation and subcloning of the DNA fragment containing the *lysU* upstream region. Plasmid pFU2 was constructed by isolating the 3.3-kb *Eco*RI fragment from pFN120 (4) and ligating it into the *Eco*RI site of pBlueScript$^+$ (pBS$^+$). Plasmid pFU2 was then digested with *Dra*I, producing a 497-bp fragment carrying the *lysU* upstream sequence. This

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype and/or relevant characteristics	Source or reference
E. coli		
CU1008	*E. coli* K-12 *ilvA*	L. S. Williams
MEW1	CU1008 Δ*lac*	9
MEW26	MEW1 *lrp*::Tn*10*	9
MEW30	MEW1 *metK-62*	9
MEW31	MEW1 *metK-62 lrp*::Tn*10*	9
W3110	Parent strain	10
BE1	W3110 *lrp*::Tn*10* by transduction from MEW26	This work
Plasmids		
pFN120	*lysU*$^+$, Amp^r	7
pFU10	108-bp *Dra*I-*Sau*3A fragment of pFN120 cloned into pBluescript$^+$	This work
pBluescript	SK$^+$ and SK$^-$	Stratagene Co.

fragment was digested with *Sau*3A, and the resulting 108-bp fragment (corresponding to nucleotides 158 through 265 as numbered in reference 4) was ligated into pBS$^+$ that had been digested with *Bam*HI and *Eco*RV. The resulting plasmid was called pFU10. Digestion with either *Hin*dIII and *Sac*I or *Xba*I and *Xho*I produced 150- or 148-bp fragments containing 42 or 40 nucleotides from the polylinker region of pBS$^+$ in addition to the 108-bp *lysU* upstream sequence. This fragment was used for gel retardation and footprinting studies after purification from agarose gels using the MERmaid kit (Bio-101 Inc.) and end labeling with [α-^{32}P]dATP and the Klenow enzyme.

Lrp protein. The Lrp protein used in these studies was the generous gift of Joseph Calvo and Debbie Willins. The protein was purified to greater than 98% as described previously (24).

Gel shift assay. Binding of the Lrp protein to the upstream region of the *lysU* gene was detected by using the gel shift assay of Ricca et al. (20) with slight modifications. From 1 to 5 ng of end-labeled 150-bp fragment, prepared as described above, was incubated at room temperature for 10 min with 2 μg of herring sperm DNA and 0 to 400 ng of Lrp protein in 20 μl of binding buffer. Samples were loaded on a 5% polyacrylamide gel preelectrophoresed at 10 V/cm and electrophoresed under the same conditions.

DNase I footprinting. Samples (5 to 20 ng) of end-labeled 150-bp fragment, constructed as described above, were incubated with 3 μg of bovine serum albumin and 0 to 64 ng of Lrp protein in 20 μl of binding buffer (20). After 10 min, 0.4 U of DNase I in 1 μl was added, and the solution was incubated at 30°C for 2 min. The reaction was terminated by adding 20 μl of stop solution (0.6 M sodium acetate [pH 5.2], 50 mM EDTA, 1.5 μg of herring sperm DNA per μl). After ethanol precipitation, the pellet was resuspended in 6 μl of formamide loading buffer and electrophoresed through a 7% polyacrylamide–8 M urea sequencing gel.

Autoradiograms. Autoradiograms were digitized by using a cold charge coupled device camera (Star 1; Photometrics). The images were converted to optical densities on a Silicon Graphics IRIS workstation. Integrated band intensities were calculated by using an EMPRO image processing package. The procedures and equipment were as described previously (23).

TABLE 2. LRS activity in *lrp* and *metK* mutants of *E. coli* K-12

Strain	Relevant genotype	LRS (U/mg of protein)
MEW1	Parent	67
MEW30	*metK*	61
MEW26	*lrp*	246
MEW31	*lrp metK*	229

[a] Extracts were prepared from cells grown to the log phase in glucose-minimal medium and assayed for LRS as described previously (7), with the modifications noted in Materials and Methods.

RESULTS

LRS activity in *lrp* and *metK* mutants. We tested LRS activity in parental, *metK*, *lrp*, and *metK lrp* strains (Table 2). The LRS activity in the *lrp* mutant was 3.7-fold higher than that of the isogenic parent strain. The activity of the *lrp metK* double mutant was similarly elevated. In contrast, LRS activity in the *metK* mutant showed no significant change from that of the parent strain. We conclude that the *metK* mutation has no effect on activity and that the elevated levels of LRS activity seen in *metK lrp* strains are due to the *lrp* mutation.

Increased amount of the LysU gene product in an *lrp* mutant. There are two LRS enzymes in *E. coli* (6), and enzymatic assays do not distinguish between the two isozymes. To determine which isozyme is expressed at elevated levels in the *lrp* strain, two-dimensional gel electrophoresis was performed on total protein extracts of isogenic *lrp*$^+$ and *lrp* strains (Fig. 1). The boxes in Fig. 1 surround the two isozymes of LRS (6), and it is clear that the *lysU* gene product (upper spot in the box) and not the *lysS* gene product (lower spot in the box) is expressed at elevated levels in the *lrp* mutant. The differences in LRS expression evident in Fig. 1 were observed in at least six repetitions of this experiment and were also seen when the two-dimensional gel electrophoresis was performed with an *lrp* strain obtained by transduction of the *lrp*::Tn*10* allele constructed in Calvo's laboratory (16).

When *lrp*$^+$ cells were grown in the presence of 10 mM leucine, *lysU* expression was elevated, although not to the extent seen in the *lrp* strain (data not shown). This pattern of expression is consistent with Lrp acting as a repressor of *lysU*, with repression relieved when leucine is added to the medium. However, measurement of the levels of protein expression does not provide any indication of whether the regulation of *lysU* expression by the *lrp* gene product is direct or indirect.

Retardation of a fragment containing upstream DNA from a *lysU* clone. Experiments were conducted to provide preliminary evidence concerning a direct interaction of the Lrp protein with the *lysU* gene. In these studies, we used a DNA fragment containing 108 bp from nucleotide 158 through 265 in the *lysU* sequence (4) and 42 bp from the polylinker region of pBS$^+$. The translation initiation site for *lysU* is at nucleotide 316. The transcription start site has not been determined, although regions with strong homologies to −35 and −10 sequences were identified at positions 194 through 199 and 216 through 221 (4).

Purified Lrp protein retarded the 150-bp fragment (Fig. 2A). The fraction of DNA retarded increased as the amount of Lrp protein increased from 25 to 400 ng; all of the input DNA was bound when 400 ng of binding protein was added. We also examined binding of Lrp to two different segments

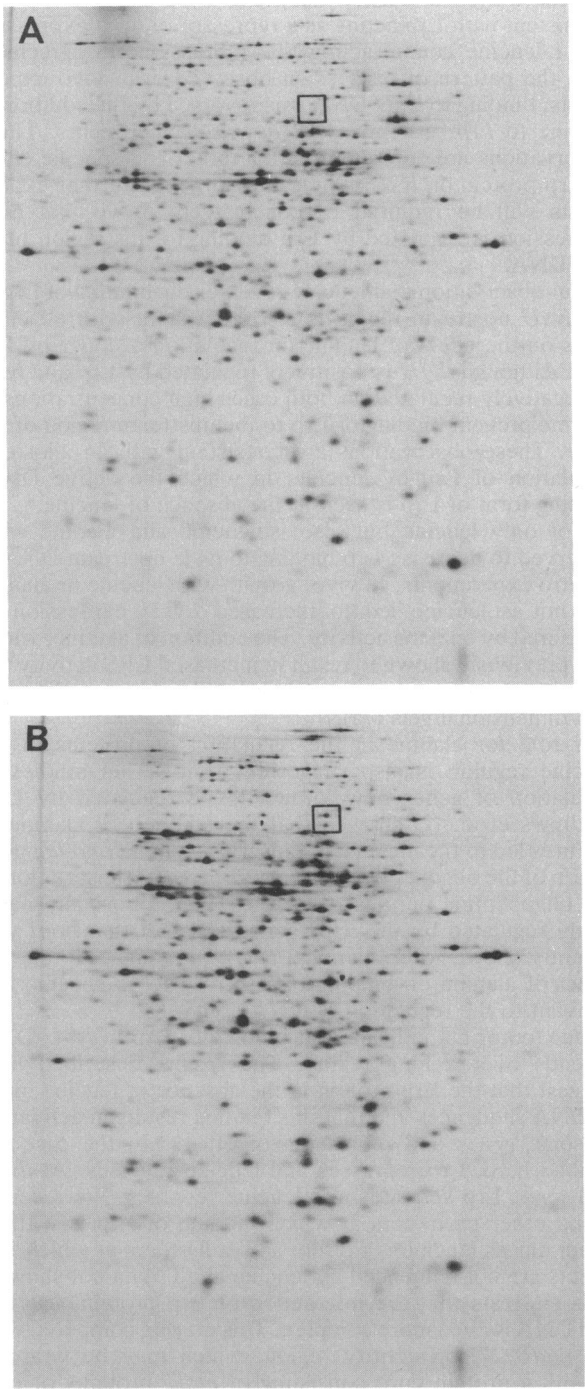

FIG. 1. Expression of individual polypeptides. Two-dimensional gel electrophoresis of proteins from isogenic Lrp^+ and Lrp^- strains were examined. Strains W3110 (A) and BE1 (W3110 *lrp*::Tn*10*) (B) were grown at 37°C in glucose-minimal morpholine propanesulfonic acid medium supplemented with thiamine and labeled for 5 min with [^{35}S]methionine during exponential growth. Polypeptides were separated by isoelectric focusing in the horizontal dimension (acidic polypeptides migrate to the right) and by electrophoresis in the presence of sodium dodecyl sulfate in the vertical direction (small polypeptides migrate to the bottom). The square on each autoradiogram indicates the polypeptides of LRS form I (lower spot, the *lysS* gene product) and form II (upper spot, the *lysU* gene product) (6).

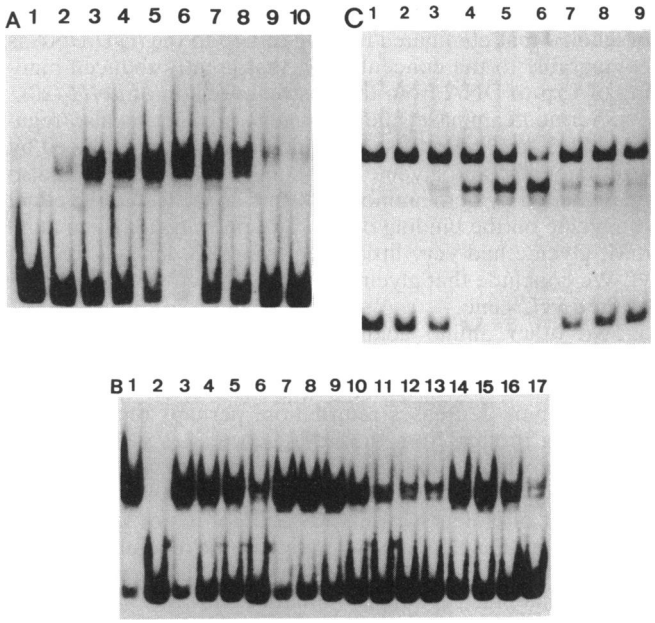

FIG. 2. (A) Gel retardation assays with Lrp protein. Lanes 1 through 6 represent incubations with 0, 25, 50, 100, 200, and 400 ng of Lrp protein, respectively. Various amounts of purified Lrp protein were incubated in a 20-μl volume with the 150-bp *Hin*dIII-*Sac*I fragment from pFU10, which carries 108 bp of *lysU* upstream sequence. Lanes 7 through 10 represent incubations with 200 ng of Lrp protein and leucine at 2, 4, 8, and 16 mM, respectively. The percentage of DNA retarded was determined from the autoradiograms as described in Materials and Methods. For lanes 1 through 10, the percentages of DNA retarded were 0, 2, 46, 43, 79, 100, 60, 52, 3, and 0%, respectively. (B) Effect of various amino acids on gel retardation assays with Lrp protein. Experiments were performed as described above for panel A with 200 ng of Lrp protein in all experiments except that in lane 2, no protein was added. Leucine (1, 2, 4, and 8 mM), glycine (10, 20, and 40 mM), L-alanine (5, 10, 20, and 40 mM), and L-isoleucine (4, 8, 16, and 32 mM) were added to lanes 3 through 17, respectively. The percentages of DNA retarded were 90, 0, 80, 55, 43, 24, 83, 73, 60, 30, 14, 9, 9, 47, 44, 19, and 5% for lanes 1 through 17, respectively. (C) Gel retardation assays on a longer *lysU* sequence. A purified 497-bp *Dra*I fragment was digested with *Sau*3A, producing the 108-bp fragment used for the experiments shown in panels A and B, a 7-bp fragment which is not visible, and a 382-bp fragment downstream of the 108-bp fragment and including some of the coding sequence. Lanes 1 through 6 represent incubations with 0, 50, 100, 200, 300, and 400 ng of Lrp protein, respectively. Lanes 7 through 9 represent incubations with 400 ng of Lrp protein and leucine at 5, 10, and 20 mM, respectively.

of the *lysU* sequence. We isolated the entire *Dra*I 497-bp fragment as described above for the smaller fragment, digested it with *Sau*3A, and studied the retardation of the digested mixture. The 108-bp fragment was retarded by Lrp, but the larger fragment was not (Fig. 2C).

If the binding of Lrp protein to DNA from the upstream region of *lysU* represents a specific interaction of Lrp protein with this DNA and if binding is directly related to regulation of gene expression by Lrp protein, one might expect that the complex would not be formed in the presence of L-leucine. In Fig. 2A, lanes 7 through 10 show the effect of adding increasing concentrations of leucine to incubations of the 150-bp fragment with 200 ng of the Lrp protein preparation described above. The addition of 4 mM leucine decreased binding of Lrp to the DNA noticeably; when 16 mM leucine

was added, no retardation could be seen. The concentration of leucine that eliminated binding of Lrp to the *lysU* DNA is comparable to the concentration that greatly reduced binding of Lrp to DNA from the upstream region of *ilvIH* (20).

L-Serine deaminase, the *sdaA* gene product, is also regulated by Lrp (9). Because L-serine deaminase is induced by both L-leucine and glycine, we thought that *lysU* might also be regulated by both amino acids, and so we tested the effect of glycine on the binding of Lrp protein to *lysU*. Even at 40 mM, glycine had very little effect (Fig. 2B, lanes 7 through 9). We conclude that glycine is not an effector of expression of the *LysU* gene.

Two other amino acids, L-alanine (Fig. 2B, lanes 10 through 13) and L-isoleucine (lanes 14 through 17), also affected retardation of the 150-bp fragment by Lrp. The fact that L-alanine decreases retardation, perhaps removing the repressing protein from the DNA, correlates well with the fact that L-alanine induces LRS activity (6) and increases *lysU* expression in two-dimensional gels (data not shown). The effect of isoleucine on LRS activity is not known, but the addition of isoleucine and valine to the medium does not lead to increased expression of *lysU* as analyzed by two-dimensional electrophoresis (data not shown).

DNase I footprinting of Lrp protein binding to DNA upstream of *lysU*. To locate the region upstream of *lysU* to which Lrp binds, we carried out DNase I protection experiments with the 150-bp sequence and Lrp protein. Figure 3 shows the footprints obtained for each of the DNA strands in the presence of Lrp protein. The protein protected several regions of the DNA between 157 and 265 bp in the *lysU* coding region (compare, for instance, lanes 2 and 5 in Fig. 3A, which contain 0 and 8 ng of Lrp, respectively, or lanes 2 and 9 of Fig. 3B, which contain 0 and 64 ng of Lrp, respectively). The concomitant addition of leucine substantially decreased the protection observed (Fig. 3A, lanes 5 and 8; Fig. 3B, lanes 9 and 10).

To determine whether subregions of the DNA protected by Lrp from DNase I cleavage showed different affinities for Lrp, we varied the amount of Lrp incubated with the noncoding strand from 0 to 64 ng (Fig. 3B, lanes 2 through 9). We did not obtain evidence for differential affinity of Lrp for subsites within the region, but rather we observed a concentration-dependent increase in protection over the entire region.

Figure 3C compares the patterns of protection seen on the coding and noncoding strands. The protected areas do not correspond exactly on the two strands but overlap for most of their length. The regions separating the protected areas showed a regular pattern of alternately increased and decreased sensitivity to cleavage, as was reported for the MalT activator binding to regions upstream of *malE* and *malK* (19).

DISCUSSION

In this paper we provide evidence that expression of the *lysU* gene is regulated by the *lrp* gene product, thus including *lysU* among the genes now known to constitute the leucine regulon of *E. coli*. This is indicated by the fact that LRS activity increased fourfold in an *lrp* mutant and that this corresponded to an actual increase in the *lysU* gene product as identified on two-dimensional gels.

Gel retardation and DNase I footprinting experiments demonstrate that purified Lrp protein binds to the DNA sequence immediately upstream of the *lysU* gene and may exert its effect on *lysU* directly. The pattern of expression of *lysU* seen by two-dimensional gel electrophoretic analysis is consistent with Lrp acting as a repressor of *lysU* expression and L-leucine counteracting this repression. In agreement with the pattern of expression observed in in vivo experiments, binding to *lysU* DNA was reversed by the addition of leucine to *Lrp* protein in our in vitro experiments. These observations are consistent with a direct and specific effect of Lrp protein on *lysU* expression, although further experiments will be required to conclusively prove that *lysU* expression is regulated by Lrp binding to this region of *E. coli* DNA.

Our observation that leucine reverses the binding of Lrp to the *lysU* upstream region is also consistent with observations on the effect of leucine on regulation of *ilvIH* by Lrp (16). Although *ilvIH* is positively regulated by Lrp and *lysU* is negatively regulated, in both cases high concentrations of leucine prevent binding of Lrp to the upstream region of the gene. These observations are consistent with an allosteric regulation of Lrp by leucine, in which the active DNA-binding form of Lrp is seen in the absence of leucine.

Not only leucine but also isoleucine and alanine were observed to decrease Lrp binding to *lysU* upstream DNA in in vitro experiments. In vivo, growth with leucine or alanine but not isoleucine led to increased *LysU* expression as measured by enzyme activity. The addition of alanine, which was previously shown to result in increased LRS activity (6), also leads to elevated synthesis of LysU as judged by two-dimensional gels (7a).

A role for alanine in the regulation of proteins in the leucine regulon is also suggested from earlier studies of regulation of genes now known to be regulated by Lrp. Andrews et al. (2) showed that the addition of alanine to cultures led to the increased transcription of an *opp-lac* gene fusion of the oligopeptide permease operon. Transcription of the oligopeptide permease operon (1, 2) is normally negatively regulated by the *oppI* gene. The fact that *oppI* was recently shown to be identical to *lrp* (24) suggests that the effect of alanine on binding of Lrp to *lysU* is specific and relevant to the regulation of *lysU* in vivo.

Our footprints indicate that binding of Lrp to *lysU* DNA extends over a long region. The results described here suggest that the Lrp protein in the absence of leucine binds to DNA sequences and that this binding results in activation of some genes and repression of others. In the case described here, Lrp represses and leucine reverses. At *ilvIH* and *serA*, Lrp activates and leucine reverses. We recently found other promoters at which binding of Lrp is without effect unless leucine is present and still others at which Lrp effects are not influenced by leucine at all (data not shown). This suggests that the interaction of Lrp protein, leucine, and DNA is even more complex. This may be compared with the *malB* 271-bp control region, which may be wrapped around a protein core consisting of *malT* proteins or of a mixture of *malT* and cyclic AMP receptor protein (19). Raibaud suggested that the structure of certain regulatory proteins may permit the formation of oligomers, some made up of identical subunits and others made up of subunits from more than one regulatory protein (18).

Lrp, together with leucine, coordinates expression of the genes of the leucine regulon, many of which are involved in amino acid metabolism (9). These include *ilvIH*, *serA*, *sdaA*, *oppI*, and *lysU* (9, 16). It also regulates threonine dehydrogenase activity. Strains with a *metK* mutation, which results in decreased synthesis of *S*-adenosylmethionine synthetase, rapidly accumulate secondary mutations in *lrp* for reasons

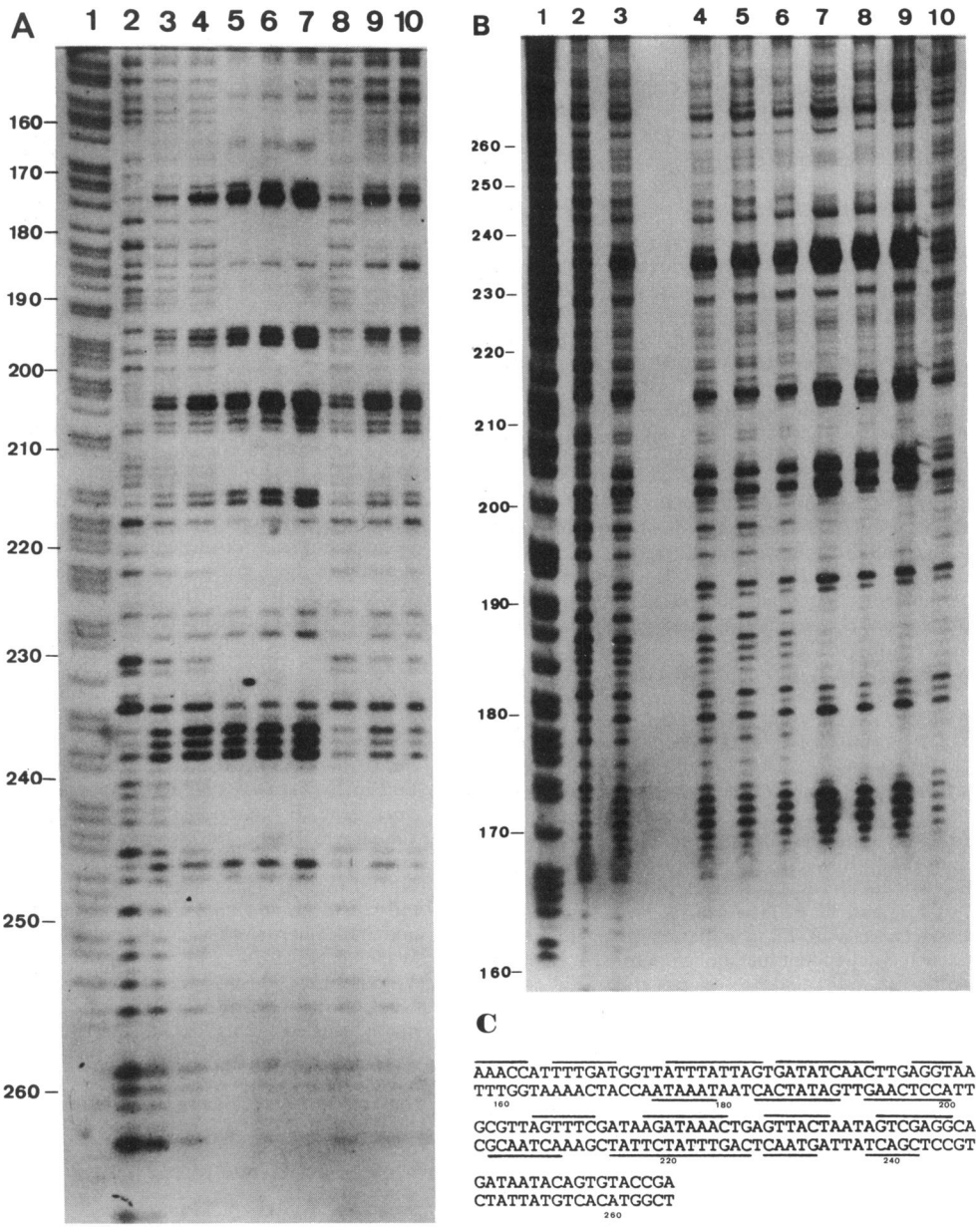

FIG. 3. DNase I footprint of Lrp protein bound to the upstream region of *lysU*. (A) Digestion pattern of the coding strand of the *Xba*I-*Xho*I fragment labeled on the *Xba*I end. Lane 1 shows an A+G sequencing ladder. Lanes 2 through 7 represent incubations with 0, 2, 4, 8, 16, and 32 ng of Lrp protein, respectively. Lanes 8 through 10 represent incubations with 8, 16, and 32 ng of LRP, respectively, in the presence of 20 mM leucine. (B) Digestion pattern of the noncoding strand of the *Hin*dIII-*Sac*I fragment labeled on the *Hin*dIII end. Lane 1 shows an A+G sequencing ladder. Lanes 2 through 9 represent incubations with 0, 2, 4, 6, 8, 16, 32, and 64 ng of Lrp protein, respectively. Lane 10 represents an incubation with 64 ng of Lrp protein and 16 mM L-leucine. (C) Comparison of sites protected by Lrp protein on the two strands of DNA upstream of *lysU*. Solid lines are drawn under or over protected nucleotides. This fragment runs from nucleotides 158 to 265 of the original clone (4), on which the Shine Delgarno sequence was at nucleotides 304 to 309 and the first translated codon was at nucleotide 316.

that are not yet understood (9). Our two-dimensional gels indicate that the levels of other proteins are also affected in *lrp* strains.

The leucine regulon thus affects a large portion of *E. coli* metabolism. The enzymes affected are consistent with our earlier suggestion that leucine is an indicator to the cell that environmental conditions favor degradation of compounds available in the external medium, particularly those that supply nitrogen (12). We hope that identification of the proteins regulated by Lrp will allow us to determine the physiological role of this system with more specificity.

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