JOURNAL OF BACTERIOLOGY, Dec. 1996, p. 7234–7240 Vol. 178, No. 24
0021-9193/96/$04.00+0
Copyright © 1996, American Society for Microbiology

Lrp Is a Direct Repressor of the *dad* Operon in *Escherichia coli*

ELIZABETH MATHEW,† JIZU ZHI, AND MARTIN FREUNDLICH*

Department of Biochemistry and Cell Biology, State University of New York, Stony Brook, New York 11794

Received 7 August 1996/Accepted 8 October 1996

Expression of the degradative D-amino acid dehydrogenase (*dad*) operon is known to be increased when *Escherichia coli* is grown in the presence of D- or L-alanine. Alanine is thought to act as an inducer to block the action of a postulated repressor. This operon is also believed to be regulated by catabolite repression. We have used in vivo and in vitro experiments that show that the *dad* repressor is the leucine-responsive regulatory protein (Lrp). *dad* expression in a *dad-lacZ* operon fusion strain was increased four- to sevenfold when cells were grown in minimal medium containing alanine or leucine. A strain lacking Lrp had high-level constitutive *dad* expression. Gel retardation and footprinting studies revealed that Lrp binds in vitro to multiple sites over a large area in the *dad* promoter region. This binding was reduced by alanine or leucine. In vitro transcription assays, using a plasmid template and primer extension analysis, identified three major *dad* transcripts (Tr1, Tr2, and Tr3). The formation of these transcripts was differentially regulated by cyclic AMP-cyclic AMP receptor protein complex, and each was strongly repressed by Lrp. Alanine or leucine completely (for Tr1 and Tr2) or partially (for Tr3) reversed Lrp inhibition. Site-directed mutagenesis of an Lrp binding site strongly reduced Lrp binding and prevented Lrp repression of *dad* transcription in vivo and in vitro. Taken together, these results strongly suggest that Lrp and alanine or leucine act directly to repress and induce, respectively, transcription of the *dad* operon.

The D-amino acid dehydrogenase (*dad*) operon is required by *Escherichia coli* for growth on L- or D-alanine as its sole carbon and energy source (33). This operon contains two structural genes, *dadA*, which codes for one subunit of D-amino acid dehydrogenase, and *dadX*, which encodes the catabolic alanine racemase (32). The racemase converts L-alanine to the D isomer, which is then oxidatively deaminated by the D-amino acid dehydrogenase to pyruvate and ammonia (6).

Expression of *dad* is increased when either L- or D-alanine is present in the growth medium (34). However, L-alanine is thought to be the actual activator because D-alanine does not increase *dad* expression in strains that lack alanine racemase activity due to mutations in *dadX* (32). It has been suggested that L-alanine acts as an inducer by antagonizing a putative *dad* repressor (15). This operon has also been reported to be positively regulated by cyclic AMP-cyclic AMP receptor protein complex (cyclic AMP-CRP) (6). Consistent with this is a recent report showing that cyclic AMP-CRP binds in vitro to two areas in the *dad* promoter region (18).

The leucine-responsive regulatory protein (Lrp) is a regulatory factor that controls, either positively or negatively, the expression of many genes, including numerous operons involved in amino acid metabolism (reviewed in references 3 and 21). In most cases, Lrp regulation of gene activity either requires or is antagonized by leucine. In some systems, alanine as well as leucine alters Lrp action (5, 17). In vitro studies have shown that Lrp binds to multiple sites over a large region upstream of Lrp-regulated promoters and that this binding is modulated by leucine or alanine (17, 30). Analyses of some of these sites by mutagenesis have provided evidence that they function in vivo and suggest that Lrp binding to promoter regions directly alters operon activity (9, 25, 30, 31). This conclusion was strengthened by results which showed that the positive action of Lrp on *ilvIH* expression in vivo could be duplicated in a purified in vitro transcription system (35).

In the present work we demonstrate that Lrp is a direct repressor of the *dad* operon. An *lrp* deletion strain was constitutive for *dad* expression. In vitro, Lrp bound to multiple sites in the *dad* promoter region and mutagenesis showed that this binding was necessary for the negative effect of Lrp on *dad* expression in vivo and in vitro. In addition, Lrp strongly blocked *dad* transcription in a purified in vitro system and this inhibition was reversed by alanine or leucine, the in vivo inducers of the *dad* operon.

* Corresponding author. Mailing address: Department of Biochemistry and Cell Biology, State University of New York, Stony Brook, NY 11794-5215. Phone: (516) 632-8562. Fax: (516) 632-8575. Electronic mail address: mfreundlich@ccmail.sunysb.edu.

† Present address: Department of Pediatrics, State University of New York, Stony Brook, NY 11794-8111.

MATERIALS AND METHODS

Bacteria and plasmids. The bacteria and plasmids used in this work are listed in Table 1.

Genetic transduction using P1 vir. Transductions were carried out as described by Miller (19). Strain EC1051 was transduced to *lrp* by utilizing CV1008 as the donor strain. After transduction, Luria agar (19) containing tetracycline (15 μg/ml) was used to select for *lrp* colonies. The mutation in *lrp* was tested by the inability of the mutant to utilize glycine as a source of nitrogen (20).

β-Galactosidase assay. β-Galactosidase assays were done at 22°C as described by Miller (19). A unit of activity is defined as A_{420} per ml of cells per 20 min multiplied by 10.

Gel mobility shift assay. Gel mobility shift assays were performed essentially as previously described (8). The *Eco*RI-*Bam*HI *dad* fragment from pBdad1 was labeled with [α-^{32}P]dGTP by using the Klenow fragment of DNA polymerase. Approximately 200 pg of the fragment was incubated for 20 min at room temperature with concentrations of Lrp (about 98% pure) varying from 20 to 100 nM in a total volume of 10 μl in the presence of 12 mM Tris-HCl (pH 8.0), 5 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 6% glycerol, 0.1 mM dithiothreitol, 150 μg of bovine serum albumin (BSA) per ml, and 5.5 μg of poly(dI · dC) per ml. L-Alanine and L-leucine, when required, were added to a final concentration of 30 mM. The samples were then loaded on a 5% nondenaturing polyacrylamide gel, prepared in 0.5× TBE buffer (27), which had been preelectrophoresed for 25 min prior to loading. The Lrp used, Lrp-39, contains a tag of six histidines and was a gift from Joseph Calvo.

Footprinting. The DNase I protection assay was a modified version of the method of Schmitz and Galas (28). The *Eco*RI-*Bam*HI *dad* fragment from pBdad1 was isolated and labeled with [α-^{32}P]dGTP by using the Klenow fragment of DNA polymerase. The incubation was done essentially as described above except 2 ng of fragment was used. DNase I was then added to a final

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference or source
CV1008	*lrp*	23
DH5αF′	F′/*endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 recA1 gyrA* (Nal^r) *relA1* Δ(*lacZYA-argF*)*U169 deoR* (φ80*dlac*Δ(*lacZ*)*M15*)	New England Biolabs
EC1051	*dadA279*::Mu(Ap^r *lac*)*araD139* Δ*lac169 met1 thi trp strA*	34
ELM1541	EC1051 *lrp*::Tn*10*	This study
MC4100	F^- *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB-5301 ptsF25 deoC1 thiA1*	26
JZ100	MC4100 containing plasmid pJ490	This study
JZ101	MC4100 containing plasmid pJ308	This study
pBluescript II KS^-	Amp^r	Stratagene
pBdad1	*Cla*I-*Bst*EII *dad* promoter fragment from pBSD1 cloned into the *Eco*RI-*Bam*HI site of pBluescript II KS^-	This study
pBSD1	Contains the *dad* regulatory region, *dadA*, and part of the *dadX* structural gene	18
pJEL170	Low-copy-number plasmid	29
pELM308	pELM490 with a 2-bp substitution in a downstream Lrp binding site of the *dad* promoter region at +10 and +11	This study
pELM490	*Eco*RI-*Bam*HI *dad* promoter fragment from pBdad1 subcloned into pLSZ13	This study
pLSZ13	Plasmid designed for in vitro transcription	29
pJ490	*Eco*RI-*Bam*HI *dad* promoter fragment from pBdad1 subcloned into pJEL170	This study
pJ308	pJ490 with a 2-bp substitution in a downstream Lrp binding site of the *dad* promoter region at +10 and +11	This study

concentration of 1.4 pg/μl, and cutting proceeded at room temperature for 30 s. The reaction was stopped by the addition of 3.5 μl of 0.25 M EDTA along with 250 μg of *Saccharomyces cerevisiae* tRNA. After ethanol precipitation and drying, the samples were resuspended in 5 μl of 98% formamide dye, electrophoresed on a 5% acrylamide denaturing gel, and visualized by autoradiography.

Hydroxyl radical footprinting was done essentially as described previously (16). The top and bottom strands of the *Eco*RI-*Bam*HI *dad* fragment from pBdad1 were labeled with [α-^{32}P]dGTP and [α-^{32}P]dATP, respectively, by using the Klenow fragment of DNA polymerase. Each fragment (2 ng) was incubated at room temperature in a total volume of 50 μl with 0 or 10 nM Lrp in the following buffer: 40 mM Tris-HCl (pH 8.0), 70 mM KCl, 7 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM ATP, and 100 μg of BSA per ml. After 15 min, 20 μl of cutting mixture [20 mM sodium ascorbate, 0.6% H_2O_2, 0.2 mM $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$, 0.8 mM EDTA] was added and cutting proceeded at room temperature for 2 min. The reaction was stopped by the addition of 40 μl of 0.1 M thiourea and 12 μl of 0.4 M EDTA. The samples were then ethanol precipitated in the presence of 0.3 M sodium acetate and 10 μg of yeast tRNA, dried, resuspended in 5 μl of 98% formamide dye, and loaded on a 5% acrylamide denaturing gel.

RNA isolation. RNA was isolated essentially as described by Ausubel et al. (2) except a phenol-chloroform extraction was done before ethanol precipitating the samples and the RNA was washed three times with 70% ethanol before drying. DH5αF′ transformants, transformed with pELM490, were grown to an optical density at 600 nm of 0.6 to 0.9 in 20 ml of Luria broth (19) containing 100 μg of ampicillin per ml and, where indicated, 5 mM L-alanine.

In vitro transcription. Plasmids pELM490 and pELM308 were used as templates for in vitro transcription, which was done essentially as described previously (29). Plasmids were used as templates because linear fragments produced very little transcription. The templates (50 to 100 ng) were incubated in a final volume of 50 μl with or without 50 nM Lrp for 10 min at 37°C in the following buffer: 40 mM Tris-HCl (pH 8.0), 70 mM KCl, 7 mM $MgCl_2$, 1 mM dithiothreitol, and 0.1 mM ATP. L-Alanine and L-leucine, when used, were added to a final concentration of 30 mM. CRP (40 nM), cyclic AMP (50 μM), and RNA polymerase (0.02 U/μl) (United States Biochemical) were then added, and incubation continued for 10 min at 37°C. The polymerization reaction was started by the addition of nucleoside triphosphates (130 μM) and 10 μg of rifampin per ml. The reaction was stopped after 10 min by phenol-chloroform extraction followed by ethanol precipitation in the presence of 0.3 M sodium acetate (pH 7.0). After washing with 70% ethanol and drying, the pellet was resuspended in 8 μl of annealing buffer (125 mM KCl, 10 mM Tris-HCl [pH 8.3]).

Primer extension. Primer extension was done by a modification of a procedure obtained from Carol Greider (10). The primer (2 μg) that was used for the extension reaction was end labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase as described by Sambrook et al. (27) and was resuspended in 40 μl of RNase-free water. The in vitro or in vivo RNA was allowed to anneal with 2 μl of end-labeled primer for 5 min at 65°C and 30 min at 42°C, and this was followed by a 15-min room temperature incubation. A total of 2 μl of deoxynucleoside triphosphates (2.5 mM), 0.4 μl of avian myeloblastosis virus reverse transcriptase (2 to 4 U), and 0.9 μl of 5× avian myeloblastosis virus reverse transcriptase buffer were added to 2.7 μl of the annealed mixture and allowed to incubate at 42°C for 45 min. The reaction was stopped by the addition of 5 μl of 98% formamide dye. After heat denaturation, the samples were loaded and run on a 5% acrylamide denaturing gel. Visualization was performed by autoradiography.

Site-directed mutagenesis. Mutagenesis was done with pBdad1 by utilizing the PCR as described previously (12) with two back-to-back primers, M308 (5′ GTAATCTAATCACATCTTGAT 3′) and M309 (5′ GATTCTTTTACTGTAT CTACC 3′), both of which have a mismatch with the template at the 5′ end. M308 and M309 anneal to the regions of the *dad* promoter from −11 to +10 and +11 to +31, respectively. The mutagenesis reaction mixture contained 10 ng of template, 100 pmol of each primer, 1 U of Vent polymerase (New England Biolabs), the four dNTPs (200 nM each), and 1× Vent polymerase buffer in a total volume of 100 μl. The mixture was denatured at 94°C for 1 min and allowed to anneal at 50°C for 1 min, and the extension reaction proceeded for 4 min at 72°C for a total of 30 cycles. The amplified plasmid band was purified from a low-melting-point agarose gel, phosphorylated, self-ligated, and used to transform competent DH5αF′ cells. Colonies that contained the ligated plasmid were selected for on Luria agar (19) containing 100 μg of ampicillin per ml. Plasmids were isolated from a number of transformants, and the presence of the mutation was confirmed by sequencing with the Sequenase 2.0 sequencing kit (United States Biochemical) used according to the manufacturer's instructions.

RESULTS

Lrp inhibits *dad* expression in vivo. These experiments were initiated because of our observation that strains lacking integration host factor grow very poorly in media containing alanine as the sole carbon source (7). Since the *dad* operon is required for alanine catabolism (32), we began an analysis of factors affecting *dad* expression, using the *dad-lacZ* operon fusion strain EC1051 (34). As has been previously reported (34), *dad* expression was strongly increased if alanine was included in the growth medium (Fig. 1A). A comparable increase in the level of β-galactosidase activity was found when EC1051 was grown in minimal medium containing leucine or leucine plus alanine (Fig. 1A), suggesting that these amino acids may act on *dad* expression by the same or very similar mechanisms. Growth in the presence of numerous other amino acids (2 mM), including methionine, glycine, serine, threonine, isoleucine, and valine, did not appreciably increase the level of β-galactosidase activity (36).

The in vivo expression of a number of operons in *E. coli* has been found to be affected by alanine and leucine, and this has been correlated with the function of the leucine-responsive regulatory protein (Lrp) (3, 21). To examine whether *dad* is regulated by Lrp, we transduced a deletion of *lrp* into strain EC1051 and measured β-galactosidase levels in the mutant

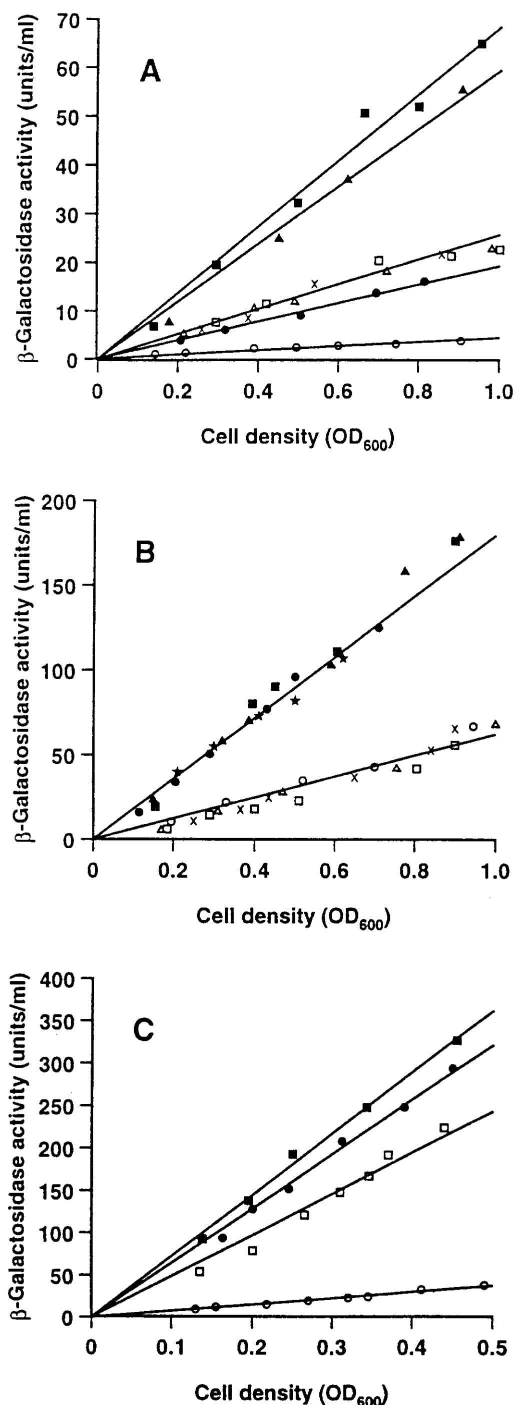

FIG. 1. *dad* expression in strains containing a *dad-lacZ* operon fusion. Cells were grown in minimal medium with the indicated carbon source with or without 2 mM L-alanine or L-leucine. At the indicated cell densities (OD_{600} [optical density at 600 nm]), samples were removed and β-galactosidase activity was determined as described in Materials and Methods. (A) EC1051 (*lrp*$^+$). Open symbols, glucose as carbon source; closed symbols, glycerol as carbon source. Symbols for amino acids added: circles, none; squares, alanine; triangles, leucine; multipliers, alanine plus leucine (glucose grown). (B) ELM1541 (*lrp*::Tn*10*). Open symbols, glucose as carbon source; closed symbols, glycerol as carbon source. Symbols for amino acids added: circles, none; squares, alanine; triangles, leucine; multipliers, alanine plus leucine (glucose grown); stars, alanine plus leucine (glycerol grown). (C) JZ100 with the Lrp site intact (open symbols) and JZ101 with the Lrp site mutated (closed symbols). The cells were grown in minimal-glucose medium without (circles) or with (squares) alanine.

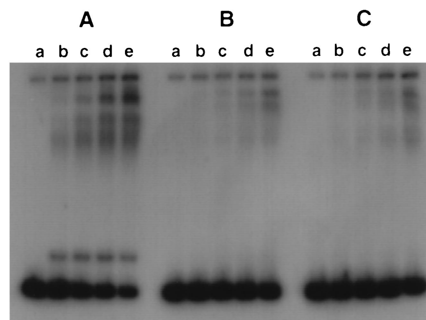

FIG. 2. Gel retardation analysis of Lrp binding to the *dad* promoter region. (A) An end-labeled *dad* promoter fragment was incubated with the indicated amounts of Lrp and the DNA and the DNA-protein complexes were separated on a polyacrylamide gel as described in Materials and Methods. Poly(dI · dC) was added to prevent nonspecific binding of Lrp to the DNA. Lanes contained Lrp at concentrations (nM) as follows: a, 0; b, 25; c, 50; d, 75; e, 100. The amount of Lrp required for 50% binding was approximately 80 nM. The band at the top of the gel corresponds to material remaining in the well. (B and C) Same as panel A except that 30 mM L-leucine (B) or 30 mM L-alanine (C) was added just prior to the addition of Lrp.

and parent grown under various conditions. The data in Fig. 1B show that the level of *dad* expression in the mutant is approximately 10- to 15-fold higher than that in the parent when cells are grown in minimal medium. Significantly, this high-level expression was not increased when the *lrp* deletion strain was grown in the presence of added alanine or leucine (Fig. 1B). Taken together, these data suggest that Lrp represses *dad* and that alanine and leucine act positively on *dad* expression by blocking Lrp action.

Lrp binds to multiple sites in the *dad* promoter region. We used DNA mobility shift assays to study possible direct interactions of Lrp, alanine, and leucine with the *dad* promoter region. The results in Fig. 2A show binding of purified Lrp to a 416-bp *Eco*RI-*Bam*HI *dad* promoter fragment. The presence of numerous Lrp-DNA complexes suggests that Lrp binds to multiple sites on this fragment. The addition of alanine or leucine reduced the ability of Lrp to bind to *dad* DNA (Fig. 2B and C).

To further analyze Lrp binding to *dad*, the DNA fragment used in the mobility shift assay was used in footprinting experiments. The data in Fig. 3A show that Lrp protects or alters cutting by DNase I over the entire *dad* fragment. The region from −38 to +22 shows a more classic DNase I protection pattern, in which most of the bases were protected by Lrp from cleavage. In the remainder of the fragment there are periodic regions of protection and hypersensitivity. Although not as dramatically as in the mobility shift experiment, alanine or leucine substantially altered the pattern of Lrp binding. This was especially apparent in the region from +18 to +100.

In an attempt to better define the contact points between Lrp and the *dad* promoter region, we used hydroxyl radical footprinting, a technique that, in contrast to DNase I footprinting, uses a small reagent for cleaving DNA (16). The results in Fig. 3B show that Lrp protects the DNA from cleavage in a regular pattern of alternating protected and unprotected bases from bp −168 to +13. The protected regions of approximately 5 bp are separated by approximately 6 to 8 bp of unprotected DNA. This large area of interaction and the pattern of protection are similar to results reported for Lrp binding in a number of other promoter regions (9, 22, 30). It has been suggested that these binding characteristics indicate that Lrp causes the DNA to be looped or wrapped around a core of Lrp molecules (30).

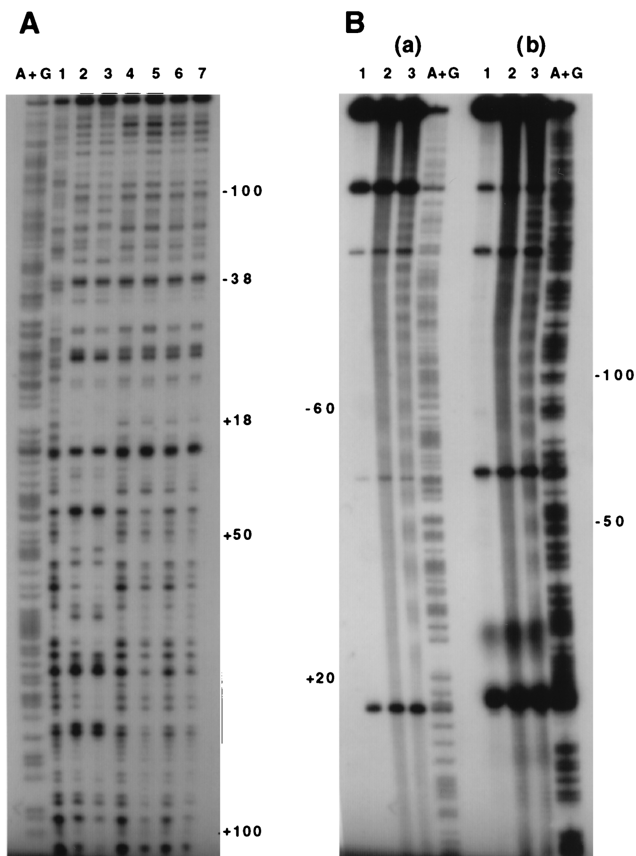

FIG. 3. Footprinting analysis of Lrp binding to the *dad* promoter region. (A) DNase I protection. The top strand of the DNA fragment used in Fig. 2 was used for DNase I footprinting. Lanes contained Lrp at concentrations (nM) as follows: lane 1, 0; lanes 2, 4, and 6, 10; lanes 3, 5, and 7, 20. L-Leucine (30 mM) was added in lanes 4 and 5, and L-alanine (30 mM) was added in lanes 6 and 7. Lane A + G contains the A + G sequencing ladder. The sequence is numbered as shown in Fig. 4. (B) Hydroxyl radical protection. The DNA fragment was the same as that used in Fig. 2. (a) Top strand; (b) bottom strand. Lanes: 1, uncut DNA; 1 and 2, no Lrp added; 3, 10 nM Lrp; 4, A + G sequencing ladders. The prominent bands seen in all of the lanes are probably DNA fragments formed by the nicking of the DNA during isolation.

Site-directed mutagenesis alters Lrp binding in vitro and strongly reduces Lrp repression in vivo. We used site-directed mutagenesis to evaluate the physiological significance of Lrp binding in the *dad* promoter region. The T's at +10 and +11 (Fig. 4) were changed to C and G, respectively. These bases were chosen for alteration because they were protected by Lrp in footprinting experiments (Fig. 3) and they are centered in a group of 13 of 15 nucleotides (Fig. 4) that are identical to a consensus sequence that has been suggested for Lrp binding to DNA (4). Hydroxyl radical footprinting (Fig. 5) showed strongly altered Lrp binding to the mutated fragment. Binding in the region from −81 to +13 was reduced, while Lrp binding further upstream did not appear to be strongly altered by the mutation.

The effect of the mutation on *dad* function in vivo was examined by cloning the mutated and wild-type fragments in front of *lacZ* in the low-copy-number plasmid pJEL170 (29). These new plasmids were used to transform MC4100, and *dad* promoter activity was determined by measuring β-galactosidase levels in cells grown in minimal-glucose medium with and without L-alanine. The results in Fig. 1C show that the level of β-galactosidase activity from the plasmid with the altered *dad*

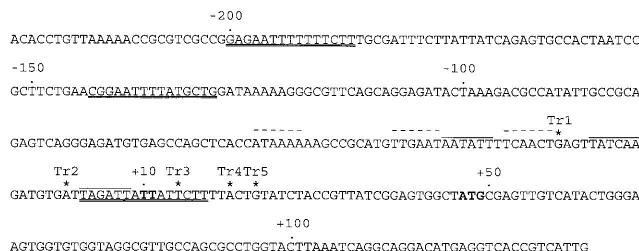

FIG. 4. Nucleotide sequence of the *dad* promoter region. The nucleotide sequence is from Lobocka et al. (18). The nucleotides are numbered from the start of transcription of transcript Tr2 identified previously in vivo (18) and in vitro, in this study (Fig. 6). Stars indicate the start of *dad* transcripts (Tr1 to Tr5), detected in vitro (Fig. 6). Possible −35 and −10 elements for the major transcripts Tr1 to Tr3 are shown as broken and solid lines, respectively, above the sequence. The ATG at +47 indicates the start of *dad* translation. The T's in boldface lettering at +10 and +11 were changed by mutagenesis to C and G, respectively, resulting in altered Lrp binding (Fig. 5). These nucleotides are within a sequence, centered at +10, which is very similar to a proposed 15-bp consensus sequence, (C/T)AGHA(A/T)ATT(A/T)TDCT(A/G), where H = not G, and D = not C (4). Two additional sequences, very similar to this 15-bp consensus, are centered at −134 and at −193. These putative sites are indicated by double underlining beneath the sequence. These sites have similarity scores (4) of 69.1, 88.2, and 76.9, respectively. These scores are higher than scores from a number of other sites which have been shown to bind Lrp (4). A sequence similar to that believed to be important for Lrp binding in the *pap* operon, Gnn(n)TTT(t) (22), is found beginning at +97, +51, +5, −16, −93, −140, −184, and −198.

promoter fragment was eightfold higher than that found in the strain with the normal fragment. In addition, this high level of activity in the mutant was not increased significantly when cells were grown in the presence of alanine. These results indicate that Lrp binding to the *dad* promoter region is required for the

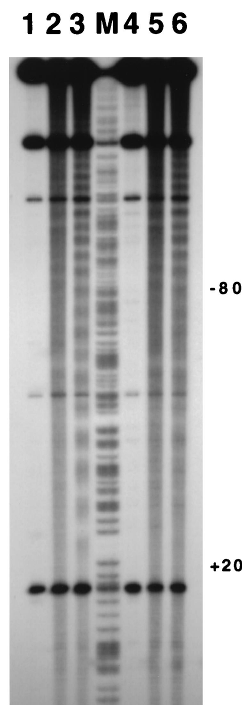

FIG. 5. Mutagenesis alters Lrp binding in the *dad* promoter region. The top strand of the fragment used in Fig. 3 was subjected to hydroxyl radical cleavage in the presence (lanes 3 and 6) or absence (lanes 2 and 5) of 10 nM Lrp. Lanes 1 to 3, wild-type fragment; lanes 4 to 6, mutated fragment. The DNA in lanes 1 and 4 was uncut free DNA. Lane M contains the A + G sequencing ladder.

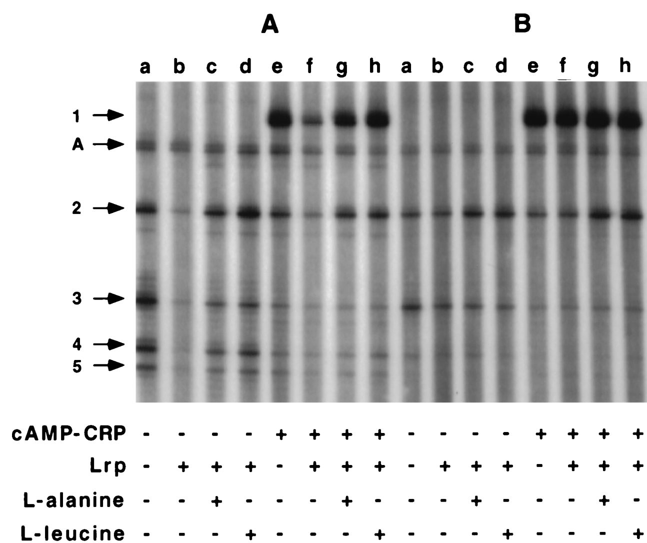

FIG. 6. *dad* transcription in vitro. (A) Repression by Lrp and effect of alanine and leucine. RNA was made in vitro with plasmid pELM490 as the template. *dad* transcripts were detected by primer extension with primer PE-1. Additions: cyclic AMP (50 μM)–CRP (40 nM), lanes e to h; Lrp (50 nM), lanes b to d and f to h; L-alanine (30 mM), lanes c and g; L-leucine (30 mM), lanes d and h. Arrows 1 to 5 indicate *dad* transcripts Tr1 to Tr5, respectively. Arrow A indicates the *amp* control transcript made from plasmid pELM490 and detected with primer Pamp. (B) Lrp repression is strongly reduced by the Lrp binding site mutation. RNA was made in vitro with plasmid pBL308 as the template. All other conditions and explanations are the same as for panel A.

negative effect of Lrp on *dad* expression in vivo. The data also suggest that Lrp binding to a site that includes bp +10 and +11 is critical for this inhibition.

Lrp represses *dad* transcription in vitro. To investigate if Lrp is a direct repressor of *dad* we analyzed *dad* expression in a purified in vitro transcription system. The *Eco*RI-*Bam*HI fragment used in the in vitro binding studies was cloned into the transcription vector pLSZ13 (29) to yield pELM490. This new plasmid was used as a template with highly purified components, and the transcription products were analyzed by primer extension using the *dad* primer PE-1 (5′ CAACGCCT ACCACACCACTTCCCAG 3′) which is at positions +62 to +86. These experiments were performed with and without cyclic AMP-CRP since this complex was reported to activate *dad* transcription in vivo (18). Five transcripts were observed which were either strongly activated (Tr1), inhibited (Tr3, Tr4, Tr5), or not appreciably altered (Tr2) by the addition of cyclic AMP-CRP (Fig. 6A). The formation of each of these transcripts was markedly inhibited when Lrp was added prior to the addition of RNA polymerase. Significantly, if alanine or leucine was added to the reaction mixture the inhibition was strongly reversed (Fig. 6A). This reversal ranged from 100% for Tr1 and Tr2 to 40 to 50% for Tr3, Tr4, and Tr5. In vitro transcription was also done with plasmid pBL308 as the template. This plasmid is the same as pELM490 except that the normal *dad* promoter region was replaced with one that has reduced Lrp binding (Fig. 5) and constitutive *dad* expression in vivo (Fig. 1) due to a 2-bp mutation at +10 and +11. The results in Fig. 6B show that in contrast to the strong inhibition by Lrp of transcription from the wild-type plasmid, Lrp had little or no inhibitory effect on Tr1, Tr2, and Tr3 when the mutant plasmid was used as the template. In these experiments, it was not possible to evaluate Lrp inhibition of Tr4 and Tr5 since these transcripts were very poorly made from the mutant template. It may be that the changes made at +10 and +11 have caused reduced promoter function for these transcripts.

Identification of *dad* transcripts made in vivo. To investigate if the *dad* transcripts made in vitro accurately reflected those made in vivo, we isolated RNA from cells containing plasmid pELM490 grown in Luria broth supplemented with L-alanine. This in vivo RNA, as well as RNA made by in vitro transcription, was used as the substrate for primer extension with primer PE-1. The data in Fig. 7 show similar patterns of *dad* transcription in vivo and in vitro. RNA from cells containing pELM490 yielded primer extension products with start sites the same as (for Tr1 and Tr2) or very similar (for Tr3, Tr4, and Tr5) to those of *dad* RNA made in vitro.

DISCUSSION

The data in this report strongly suggest that Lrp acts as a repressor to directly inhibit *dad* expression. (i) Gel retardation and DNase I and hydroxyl radical footprinting experiments showed specific binding of Lrp to multiple sites in the *dad* promoter region. (ii) A mutation that strongly reduced Lrp binding in vitro prevented Lrp repression of *dad* in vivo. (iii) Lrp blocked *dad* expression in a purified in vitro transcription system, and this repression was markedly reduced by alanine or leucine, compounds that induce *dad* in vivo. In addition, *dad* transcription in vitro was not inhibited by Lrp if the template contained an Lrp binding site mutation.

Lrp has been found to inhibit the expression of a number of operons in *E. coli* (3, 21), and in some of these cases it was suggested that this is due to direct interaction of Lrp at promoter regions (9, 22, 25, 31). This conclusion was based on studies that showed Lrp binding to these promoters and on Lrp binding site mutations that resulted in constitutive operon expression in vivo (9, 22, 25, 31). However, direct repression by

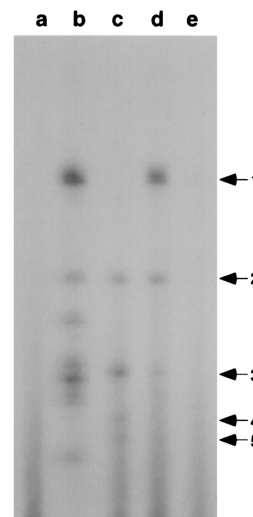

FIG. 7. Comparison of *dad* transcripts made in vivo and in vitro. For in vivo analysis, total cellular RNA was isolated from strain DH5αF′ containing plasmid pELM490 grown in Luria broth with 2 mM L-alanine. In vitro RNA was made with pELM490 as the template. In both cases, *dad* transcripts were detected by primer extension with primer PE-1. Lanes: a, primer PE-1 alone; b, primer extension products of RNA made in vivo; remaining lanes, primer extension products of RNA made in vitro in the absence of cyclic AMP-CRP (c), in the presence of 50 μM cyclic AMP and 40 nM CRP (d), and in the presence of cyclic AMP-CRP and 50 nM Lrp (e). Arrows 1 to 5 indicate *dad* transcripts Tr1 to Tr5, respectively. Tr4 and Tr5 were not detected in vivo in this experiment. The in vivo *dad* transcript seen between Tr2 and Tr3 was not found in vitro.

Lrp could only be inferred from these studies since the negative effects of Lrp on operon expression were observed only in vivo. Therefore, our in vitro transcription results constitute the strongest evidence to date that Lrp can directly inhibit transcription and that Lrp is able to accomplish this repression without the help of additional regulatory factors. Furthermore, the similarity of the data obtained in vivo and in vitro suggests that the in vitro transcription system is a reasonably accurate reflection of how *dad* is expressed and regulated by Lrp in vivo. Our results showed that, both in vivo and in vitro, transcription initiated at very similar sites, the levels of Lrp repression and alanine or leucine induction were similar, and a mutation in an Lrp binding site prevented Lrp repression.

The precise mechanism of how Lrp inhibits transcription of *dad* or of other operons is not known. However, in some of the systems examined, Lrp repression was found to require binding of Lrp to a site that overlaps the RNA polymerase binding site (9, 22). For example, in *lysU*, cooperative binding of Lrp to multiple sites has been suggested to enhance Lrp binding to a critical site for repression that encompasses the −35 region (9). It has therefore been postulated that Lrp could block transcription by directly interfering with RNA polymerase (13, 22). We have shown that Lrp binds to multiple sites in the *dad* promoter region and that a site that overlaps or is very close to the start of transcription of each of the major *dad* transcripts is essential for repression. While these results are consistent with the model described above, the mechanism for Lrp repression of *dad* could be more complex. Our results suggest that there are at least three major *dad* transcripts, each of which is repressed by Lrp and affected differently by cyclic AMP-CRP. This suggests that there are multiple *dad* promoters that can be repressed by Lrp by more than one mechanism. These mechanisms could involve interactions of Lrp with other proteins, such as cyclic AMP-CRP, that bind in the *dad* promoter region, as well as direct effects of Lrp on RNA polymerase. Mutational analysis of the putative *dad* promoters and in vitro transcription and binding studies should provide information on the molecular mechanism(s) for Lrp repression of the multiple *dad* transcripts.

The ability of alanine or leucine to strongly antagonize Lrp repression of *dad* is consistent with the positive role of these amino acids in the expression of numerous operons that are negatively regulated by Lrp (3, 21). In *lysU*, gel retardation analysis showed that alanine or leucine strongly reduced Lrp binding to the *lysU* promoter region, suggesting that these amino acids act as classical inducers (14) to antagonize Lrp repression (17). Similar experiments described in this report showed that Lrp binding to most areas in the *dad* promoter region was strongly inhibited by alanine or leucine. DNase I protection studies further showed that Lrp binding in the *dad* promoter-regulatory region was altered by these amino acids. These footprinting results also indicated that Lrp binding to the downstream region was considerably more sensitive to inhibition by alanine or leucine than was that to the upstream region. Whether this reflects differences in affinity of these sites for Lrp (22, 30) or more complex differences in their properties and functions is currently being investigated.

In addition to having effects on Lrp binding, alanine and leucine were found to very strongly reverse Lrp repression of *dad* transcription in vitro. Taken together, these in vitro results show that these amino acids act directly to induce *dad* expression and strongly suggest that they are not converted to other compounds (21) in order to alleviate Lrp repression of *dad* in vivo.

The gel retardation results and the pattern of protection in DNase I and hydroxyl radical footprinting experiments indicate that Lrp binds to multiple sites over a large area of *dad* promoter DNA. As in other systems (3), these techniques were unable to clearly identify individual Lrp binding sites. However, by in vitro mutagenesis and subsequent in vitro and in vivo analysis we found that Lrp binding to a site that includes nucleotides at +10 and +11 was essential for repression. This site contains sequences that are a 13/15 match to a 15-bp Lrp consensus sequence recently proposed by Cui et al. (4). Two similar sequences centered at −136 and −193, as well as other sequences postulated to be important for Lrp binding (22), are found in the Lrp promoter region (Fig. 4). Future experiments, including MPE-Fe(II) footprinting (30) and mutagenesis analysis, should help to assess the significance of these sequences to Lrp binding and function in the *dad* promoter region.

On the basis of primer extension analysis of RNA made in vivo, the *dad* operon was reported to be expressed by multiple promoters (18). It was suggested that multiple *dad* promoters could be necessary for complex regulation of this operon by numerous regulatory factors (18). Our results, which identified three major *dad* transcripts, each of which was regulated differently by cyclic AMP-CRP, are consistent with this suggestion. In addition, the differential effects of cyclic AMP-CRP on expression of individual *dad* transcripts may reflect, as in the *gal* operon (1), a biosynthetic as well as a degradative function for *dad*. Although the *dad* genes are not essential (32), perhaps under certain conditions the degradative alanine racemase, coded for by *dadX*, could be necessary for the optimal formation of D-alanine for biosynthetic functions. This compound is required for cell wall synthesis, and it is thought to usually be made by the biosynthetic alanine racemase, the product of the *alr* gene (24).

In addition to the results reported here for *E. coli*, a recent report suggests that Lrp is also involved in the regulation of *dad* expression in *Salmonella typhimurium* (11). This conclusion was based on increased *dad* expression in a strain containing an *lrp* point mutation. However, in contrast to our results with *E. coli*, an *lrp* deletion strain showed little or no increase in *dad* expression, and leucine, compared with alanine, was a weak *dad* inducer in all strains tested (11). While these data do not suggest an obvious mechanism for Lrp regulation of *dad*, they do indicate that in *S. typhimurium* this mechanism is different from that suggested by our results with *E. coli*. Whether or not this represents a fundamental difference in Lrp or *dad* function in these organisms requires further analysis.

ACKNOWLEDGMENTS

We are grateful to J. Krakow for purified CRP, J. Calvo for purified Lrp, and J. Fuchs and M. Lobocka for bacterial strains and plasmids.

This work was supported by grant GM17152 from the National Institutes of Health.

REFERENCES

1. **Adhya, S.** 1987. The galactose operon, p. 1503–1512. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1989. Current protocols in molecular biology. John Wiley and Sons, New York.
3. **Calvo, J. M., and R. G. Matthews.** 1994. The leucine-responsive regulatory protein, a global regulator of metabolism in *Escherichia coli*. Microbiol. Rev. **58:**466–490.
4. **Cui, Y., Q. Wang, G. D. Stormo, and J. M. Calvo.** 1995. A consensus sequence for binding of Lrp to DNA. J. Bacteriol. **177:**4872–4880.
5. **Ernsting, B. R., J. W. Denninger, R. M. Blumenthal, and R. G. Matthews.** 1993. Regulation of the *gltBDF* operon of *Escherichia coli*: how is a leucine-insensitive operon regulated by the leucine-responsive regulatory protein? J. Bacteriol. **175:**7160–7169.

6. **Franklin, F. C. H., and W. A. Venables.** 1976. Biochemical, genetic and regulatory studies of alanine catabolism in *Escherichia coli* K12. Mol. Gen. Genet. **149:**229–237.
7. **Freundlich, M., N. Ramani, E. Mathew, A. Sirko, and P. Tsui.** 1992. The role of integration host factor in gene expression in *Escherichia coli*. Mol. Microbiol. **6:**2557–2563.
8. **Fried, M., and D. M. Crothers.** 1981. Equilibria and kinetics of Lac repressor-operator interactions by polyacrylamide gel electrophoresis. Nucleic Acids Res. **9:**6505–6525.
9. **Gazeau, M., F. Delort, M. Fromant, P. Dessen, S. Blanquet, and P. Plateau.** 1994. Structure-function relationship of the Lrp-binding region upstream of *lysU* in *Escherichia coli*. J. Mol. Biol. **241:**378–389.
10. **Greider, C.** Personal communication.
11. **Hecht, K., S. Zhang, T. Klopotowski, and G. F.-L. Ames.** 1996. D-Histidine utilization in *Salmonella typhimurium* is controlled by the leucine-responsive regulatory protein (Lrp). J. Bacteriol. **178:**327–331.
12. **Hemsley, A., N. Arnheim, M. D. Toney, G. Cortopassi, and D. J. Galas.** 1989. A simple method for site-directed mutagenesis using the polymerase chain reaction. Nucleic Acids Res. **17:**6545–6551.
13. **Huisman, T. T., D. Bakker, P. Klaasen, and F. K. de Graaf.** 1994. Leucine-responsive regulatory protein, IS1 insertions, and the negative regulator FaeA control the expression of the *fae* (K88) operon in *Escherichia coli*. Mol. Microbiol. **11:**525–536.
14. **Jacob, F., and J. Monod.** 1961. Genetic regulatory mechanisms in the synthesis of proteins. J. Mol. Biol. **3:**318–356.
15. **Lambert, M. P., and F. C. Neuhaus.** 1972. Factors affecting the level of alanine racemase in *Escherichia coli*. J. Bacteriol. **109:**1156–1161.
16. **Levin, J. R., A. M. Burkhoff, and T. D. Tulius.** 1991. Using the chemistry of the hydroxyl radical to determine structural details about DNA and protein-DNA complexes. Biomethods **5:**133–143.
17. **Lin, R., B. Ernsting, I. N. Hirshfield, R. G. Matthews, F. C. Neidhardt, R. L. Clark, and E. B. Newman.** 1992. The *lrp* gene product regulates expression of *lysU* in *Escherichia coli* K-12. J. Bacteriol. **174:**2779–2784.
18. **Lobocka, M., J. Hennig, J. Wild, and T. Klopotowski.** 1994. Organization and expression of the *Escherichia coli* K-12 *dad* operon encoding the smaller subunit of D-amino acid dehydrogenase and the catabolic alanine racemase. J. Bacteriol. **176:**1500–1510.
19. **Miller, J. H.** 1992. A short course in bacterial genetics: a laboratory manual and handbook for *Escherichia coli* and related bacteria. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
20. **Newman, E. B., R. D'Ari, and R. T. Lin.** 1992. The leucine-Lrp regulon in *E. coli*: a global response in search of a raison d'être. Cell **68:**617–619.
21. **Newman, E. B., and R. Lin.** 1995. Leucine-responsive regulatory protein: a global regulator of gene expression in *E. coli*. Annu. Rev. Microbiol. **49:**747–775.
22. **Nou, X., B. Braaten, L. Blyn, L. Kaltenbach, and D. Low.** 1995. Differential binding of Lrp to two sets of *pap* DNA binding sites mediated by PapI regulates Pap phase variation in *Escherichia coli*. EMBO J. **14:**5785–5797.
23. **Platko, J. V., D. A. Willins, and J. M. Calvo.** 1990. The *ilvIH* operon of *Escherichia coli* is positively regulated. J. Bacteriol. **172:**4563–4570.
24. **Reitzer, L. J., and B. Magasanik.** 1987. Ammonia assimilation and the biosynthesis of glutamine, glutamate, aspartate, asparagine, L-alanine, and D-alanine, p. 302–320. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
25. **Rex, J. H., B. D. Aronson, and R. L. Sommerville.** 1991. The *tdh* and *serA* operons of *Escherichia coli*: mutational analysis of the regulatory elements of leucine-responsive genes. J. Bacteriol. **173:**5944–5953.
26. **Russo, F. D., J. M. Slauch, and T. J. Silhavy.** 1993. Mutations that affect separate functions of OmpR, the phosphorylated regulator of porin transcription in *Escherichia coli*. J. Mol. Biol. **231:**261–273.
27. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
28. **Schmitz, A., and D. J. Galas.** 1979. The interaction of RNA polymerase and Lac repressor with the *lac* control region. Nucleic Acids Res. **6:**111–137.
29. **Sun, L., and J. A. Fuchs.** 1994. Regulation of the *Escherichia coli nrd* operon: role of DNA supercoiling. J. Bacteriol. **176:**4617–4626.
30. **Wang, Q., and J. M. Calvo.** 1993. Lrp, a global regulatory protein of *Escherichia coli*, binds co-operatively to multiple sites and activates transcription of *ilvIH*. J. Mol. Biol. **229:**306–318.
31. **Wang, Q., J. Wu, D. Friedberg, J. Platko, and J. M. Calvo.** 1994. Regulation of the *Escherichia coli lrp* gene. J. Bacteriol. **176:**1831–1839.
32. **Wild, J., J. Hennig, M. Lobocka, W. Walczak, and T. Klopotowski.** 1985. Identification of the *dadX* gene coding for the predominant isozyme of alanine racemase in *Escherichia coli* K12. Mol. Gen. Genet. **198:**315–322.
33. **Wild, J., and T. Klopotowski.** 1981. D-Amino acid dehydrogenase of *Escherichia coli* K12: positive selection of mutants defective in the enzyme activity and localization of the structural gene. Mol. Gen. Genet. **181:**373–378.
34. **Wild, J., and B. Obrepalska.** 1982. Regulation of expression of the *dadA* gene encoding D-amino acid dehydrogenase in *Escherichia coli*: analysis of *dadA-lacZ* fusions and direction of *dadA* transcription. Mol. Gen. Genet. **186:**405–410.
35. **Willins, D. A., and J. M. Calvo.** 1992. In vitro transcription from the *Escherichia coli ilvIH* promoter. J. Bacteriol. **174:**7648–7655.
36. **Zhi, J., and M. Freundlich.** Unpublished data.