The Leucine-responsive Regulatory Protein (Lrp) from Escherichia coli

STOICHIOMETRY AND MINIMAL REQUIREMENTS FOR BINDING TO DNA*

Yuhai Cui†, Michael A. Midkiff, Qing Wang‡, and Joseph M. Calvo§
From the Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, New York 14853

Lrp (Leucine-responsive regulatory protein) regulates the expression of a number of operons in Escherichia coli. A recent study of DNA sequences recognized by Lrp established the consensus as a 15-bp sequence, YAGHAWATTWTDCCTR (Y = C/T, H = ‘not G,’ W = A/T, D = ‘not C,’ R = A/G) (Cui, Y., Wang, Q., Stormo, G. D., and Calvo, J. M. (1995) J. Bacteriol. 177, 4872–4880). Here we report the stoichiometry of Lrp binding (an Lrp dimer binds to a single binding site) and studies that define the minimal length of DNA required for binding. A double-stranded 15 mer having a sequence that closely matches the consensus does not show measurable binding to Lrp. One or two base pairs of DNA flanking each end are not sufficient for binding, but constructs having 3–5 additional base pairs (21 mer) show relatively strong binding. Single-stranded flanking DNA also contributes to strong binding. The extent of the contribution to binding is dependent upon whether the single strand is on the left or right of the double-stranded region and whether the polarity of the single-stranded DNA is 5’ to 3’ or 3’ to 5’.

The binding of Lrp in vitro to DNA upstream of fimA (4), ilvH (5), lysU (6, 7), ompC-micF (8), and pap (9) has been studied by DNase I footprinting. In each case, Lrp perturbs the structure of DNA over a region of 100 bp or more. For the case of ilvH, Lrp binds to six distinct sites, and the binding is highly cooperative to two groups of those sites (10). Lrp induces a bend of about 50° in binding to a single site (5).

A preliminary consensus sequence was derived from a comparison of 12 sites to which Lrp was shown to bind in MPE footprinting experiments (11). That consensus sequence was confirmed and extended by analyzing 63 sequences obtained using the “Selex” procedure of Tuerk and Gold (12, 13). The consensus, YAGHAWATTWTDCCTR (Y = C/T, H = ‘not G,’ W = A/T, D = ‘not C,’ R = A/G), is 15 bp in length and is palindromic in part. The central 5 bp are predominantly ATs, with the As distributed mostly on one strand and the Ts on the other.

Here we investigate further the requirements for Lrp interaction with a single binding site. We demonstrate that a double-stranded 15 mer having a sequence that closely matches the consensus does not bind Lrp and that flanking DNA sequences are required for strong binding. Surprisingly, single-stranded DNA in some cases provides the extra energy required for strong binding. In addition, we performed stoichiometry experiments that demonstrate that a single Lrp binding site binds one Lrp dimer.

MATERIALS AND METHODS

Stoichiometry Measurements—The DNA fragment used was one of the six binding sites for Lrp that is located upstream of the ilvH operon of E. coli (site 2). Two complementary single strands were chemically synthesized: 5’-TCAAGATGAAATCTGTTATCTGTCTT-3’ and 3’-TGACTTAGCTCAAAATAGCAGTATGCATGC-5’. Three μg of each strand were annealed by heating to 97°C in a 100-μl solution containing 10 mM Tris-HCl (pH 8.0), 1 mM sodium EDTA, and 100 mM NaCl followed by cooling slowly. End-labeled fragments were prepared with [α-32P]dCTP (Amersham Corp., 3000 Ci/mmol) using reverse transcriptase. Unincorporated label was removed by twice diluting the sample to 2 μl followed by concentration to 35 μl with an Amicon Centricon 10 filter. The specific activity of the DNA fragment was determined as follows. The amount of DNA in a sample was measured with a DNA fluorometer (Hofer Scientific Instruments, TKO 100) following the procedure described by the manufacturer. The amount of radioactivity in samples of DNA was determined by adding each sample to 0.35 cm2 of 8% polyacrylamide gel (previously electrophoresed at 14 V/cm for 2 h), incubating at 65°C overnight with 0.5 ml of 21% H2O2 and 17% HClO4 in a capped scintillation vial (14), and counting with 15 μl of biodegradable counting scintillant (Amersham) using a Beckman 7500 liquid scintillation counter.

Radioactive Lrp was prepared by in vitro transcription and translation in the presence of ['H]leucine. Plasmid pCV225, containing the Lrp gene downstream of a phage T7 promoter, was constructed as follows. The Lrp gene from plasmid pCV180 (15) was cut out with EcoRI and BamHI and cloned between the same sites of plasmid pYF C-0 (a derivative of pBS II SK+, in which the lac operator was deleted) (16). Plasmid pCV225 was isolated from strain CV1211 (M101/pCV225) and purified by Cesium centrifugation (17). Lrp was synthesized in a 50-μl reaction

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†Partly supported by a fellowship from the K.C. Wong Education Foundation, Hong Kong.
§Present address: Dept. of Human Genetics, University of Utah Health Sciences Center, Salt Lake City, Utah 84112.
¶To whom correspondence should be addressed: 451 Biotechnology Building, Cornell University, Ithaca, NY 14853. Tel.: 607-255-2437; Fax: 607-255-2428; E-mail: jmc22@cornell.edu.
1The abbreviations used are: Lrp, leucine-responsive regulatory protein; bp, base pairs; BSA, bovine serum albumin; DTT, dithiothreitol; dNTP, deoxyribonucleoside triphosphate; dNTP, deoxyribonucleoside triphosphate; MPE, methyldiunpropyl EDTA-Foe(i).
mixture containing 1 μg of plasmid pCV225 DNA, 134 pmol of 1-[4,5-3H]leucine (Amersham, 149 Ci/mmol), 1 μl of a solution containing a mixture of all of the amino acids except leucine (each at 1 μm), 1 μl of a solution containing T7 RNA polymerase, and 25 μl of rabbit reticulocyte extract. All of the components except leucine and DNA were from the TNT T7 coupled rabbit reticulocyte lysate system of Promega. After 90 min at 30°C, unincorporated leucine was removed by adding 40 μl of the sample through a spin column containing 250 μl of Sephadex G-50 swollen in TG20ED (10 mM Tris-HCl, pH 8.0, 20% glycerol, 0.1 mM EDTA, and 0.1 mM DTT). This column was centrifuged at 850 × g for 1 min, and the flow-through volume containing Lrp was collected. The specific activity of Lrp was calculated as the product: specific activity (1000 dpm/pmol) × Lrp concentration (pmol) × dpm/pmol of Lrp. The specific activity of the leucine had to be corrected because of the unradiolabeled leucine in the rabbit reticulocyte extract. To do this, a 25-μl sample of the reticulocyte lysate was added to 25 μl of 30% CH3OH, and after dilution to 1 ml with 20% CH3OH, the sample was passed through an equilibrated C-18 Sep-Pak cartridge. The eluate was dried under vacuum, dissolved in 100 μl of 0.1 M HCl, and assayed for amino acids with a Beckman amino acid analyzer. The efficiency of the scintillation counter for counting tritium was determined by measuring cpm for samples of [3H]leucine (149 Ci/mmol) that had been added to samples of polyacrylamide gel and treated with peroxide and perchlorate as described above.

In a typical experiment to determine stoichiometry, a 100-μl solution containing 40% glycerol, 40 mM Tris-HCl (pH 8.0), 20 μg of bovine serum albumin (BSA), 0.2 mM EDTA, 0.4 mM DTT, 100 mM NaCl, 20 mM MgCl2, and about 1.5 μmol of end-labeled site 2 was mixed with 100 μl of the sample containing tritiated Lrp. After 20 min at 20°C, the sample was distributed into five wells and fractionated by electrophoresis through a 1.5-mm 8% polyacrylamide gel for 2.5 h at 14 V/cm. The Lrp-DNA complex in each lane identified by autoradiography was excised in a volume of 0.35 cm2 and counted as described above using Beckman program 6. Channel 1 was used to measure tritium counts/min after corrections were made for spillover of 32P (approximately 4% of channel 2 counts). Channel 2 was used to measure 32P with no correction for tritium spillover necessary. Stoichiometry was determined from the following equation: (cpm·H/x-specific activity of DNA (cpm/pmol))/(cpm·32P/x·H efficiency (cpm/pdm)·specific activity of Lrp (dpm/pmol)).

The Molecular Weight of Lrp-DNA Complexes—Molecular weights were determined by electrophoresis through native polyacrylamide gels using procedures described by Orchard and May (18). The DNA used was Lrp binding site 2 that is at position 232 to 211 upstream of the E. coli ilvIH promoter (10). Binding reactions prepared as for band shift assays and containing 12 ng of Lrp and 2-4 labeled DNA were analyzed on a series of pre-run polyacrylamide gels (5–10%; 40:1 acrylamide:beisacrylamide; 15 cm × 18 cm; cast and run in 22 mM Tris borate buffer). Electrophoresis was performed in a cold room at 4°C until the bromophenol blue dye reached the bottom of the gel. Protein standards (about 1 μg of each protein, Sigma) were run in parallel. The centers of the bromophenol blue bands were marked with radioactive ink and by punching, respectively, for the Lrp-DNA complex and the protein standard lanes. Lanes containing label were dried onto paper and subjected to autoradiography, whereas lanes containing protein standards were stained with Coomassie Blue. The distance migrated by Lrp-DNA complexes and by each standard were measured and divided by the distance that the bromophenol blue in the same lane migrated, giving relative mobilities, R. The logarithm of R was plotted against the percent gel concentration for each species, and then the slope of each protein standard was plotted against its molecular weight on a log-log scale.

Overexpression in Vitro—An Lrp derivative having 12 extra amino acids at the NH2 terminus, including 6 His residues (6XHis-Lrp) was made by using the QIAexpress system (Qiagen, Inc.). The coding region of Lrp was amplified by means of the polymerase chain reaction, cloned into vector pQE30, and the resulting hybrid plasmid was transformed into strain M101X. 6XHis-Lrp was overexpressed after induction with isopropyl β-D-thiogalactoside and purified using a Ni-NTA affinity column. 6XHis-Lrp shows similar DNA binding activity as wild type (data not shown).

Following the procedure of Hager and Burgess (19), heterodimers were prepared by mixing 1 μl of 6XHis-Lrp (1 μg/ml), 3 μl of Lrp (1 μg/ml), and 20 μl of 8% guanidine hydrochloride, all in TG20ED, 0.1 mM NaCl, and incubating at room temperature for 1 h. The sample was diluted with 1 μl of TG20ED, 0.1 M NaCl, and after incubation for an additional 5 h, 1 μl was used in binding reactions. Electrophoresis was performed through 38-cm-long 8% polyacrylamide gels at 10 V/cm for 15 h at room temperature.

Oligonucleotides Used to Define Minimal Requirements for Lrp Binding—The oligonucleotides synthesized are shown in Scheme 1.

Complementary sequences F and R comprise a double-stranded 50 mer that bound Lrp most strongly from a collection of 63 sequences that were selected in vitro for binding to Lrp (Leu-19 in Ref. 12). The underlined region corresponds to the 15 bp consensus sequence that was defined as the comparison of the 63 sequences (12). All oligonucleotides were purified by polyacrylamide gel electrophoresis. To form duplex DNA, equal amounts of complementary strands were added to 100 mM NaCl, 10 mM Tris (pH 8.0), 1 mM EDTA, heated to 95°C for 5 min, and cooled slowly to room temperature.

Determination of Binding Constants for Lrp-DNA Interactions—For the primer extension experiments, the strategy and procedures of Liu-Johnson et al. (20) were used with some modifications. The Sequenase sequencing kit (U. S. Biochemical Corp.) was used for primer extension reactions. Primers were end-labeled with [y-32P]ATP and T4 polynucleotide kinase (17) and purified by ethanol precipitation after phenol/chloroform extraction. They were hybridized to their templates in a 1:2 molar ratio in 1 × Sequenase buffer by heating at 70°C for 10 min and cooling to room temperature slowly. About 50 pmol of product was mixed with 1 μl of DTT, 1 μl of Mn buffer, and 0.5 μl of Sequenase 2.0 in a total volume of 15.5 μl. Then 3.5-μl samples were transferred to four tubing containing 2.5 μl of dideoxynucleotide (ddNTP) termination mixes and incubated at room temperature for 30 min. The extension products from all four reactions were pooled, extracted with phenol/chloroform, precipitated with ethanol, and dissolved in 100 mM NaCl, 10 mM Tris (pH 8.0), 1 mM EDTA. To avoid denaturation from ethanol precipitation (21), the products were boiled for 2 min, chilled immediately on ice for 2 min, heated to 70°C for 10 min, and cooled slowly to room temperature. Binding reactions in a total volume of 40 μl were performed as described by Wang and Calvo (10) (no competitor DNA) using Lrp at final dimer concentrations of 8.3 or 16.6 nM and a final DNA concentration of 100 nM (based upon the concentration of primer). The sample was incubated at room temperature for 20 min, fractionated through an 8% polyacrylamide gel in the cold, and subjected to autoradiography. Regions of the gel containing DNA-Lrp complex and free DNA were excised, and DNA was eluted and ethanol precipitated. These preparations were fractionated on a 12% sequencing gel. The primer extension products from the four reactions were run alongside to identify the sequences. The radioactivity of each band was quantified with a Betascope blot analyzer (Betagen).

In other experiments, DNA samples were labeled and incubated with Lrp as described above except that samples were separated through 8% polyacrylamide gels in the cold to avoid denaturing short duplexes. In some cases, the amount of free DNA and Lrp-DNA complex were measured over a range of Lrp concentrations and binding constants were calculated as the concentration of Lrp at which half of the DNA existed as complex. In other cases, binding constants were determined relative to that for the double-stranded 50 mer using the equation K = (C/D) × 1/(P – C), where K is the binding constant, C and D are the band intensities of the complex and free DNA, respectively, and P is the total protein concentration. For these experiments, the DNA concentration was 5 μl, and the Lrp concentration was either 16.6 nM (Figs. 6 and 7) or 8.3 nM (Fig. 8). Where relative binding strengths are reported, the binding constant for a particular construct was divided by that for the double-stranded 50 mer construct, determined in the same experiment.

2Y. Cui and M. Calvo, unpublished results.
Results

Stoichiometry of Lrp Binding to DNA—For these studies, we chose site 2, a strong Lrp binding site located at positions -232 to -211 upstream of the E. coli ilvIH promoter (10). The sequence of site 2 closely matches the consensus sequence for Lrp binding, and Lrp binds to a 31 mer containing this sequence with a $K_d$ in the nanomolar range (12). We employed $^32$P-labeled site 2 DNA and $^3$H-labeled Lrp, both of known specific activity, and measured the amounts of each label in an Lrp-DNA complex that was isolated after electrophoresis. The results of three determinations (monomers per DNA) were 2.2, 2.06, and 2.3 (average 2.18). These results establish the empirical formula of the complex to be $D_2P_n$, where $D$ is DNA, $P$ is protein monomer, and $n$ is an integer.

Establishing the value of $n$ requires an estimate of the molecular weight of the complex. This was provided by measuring the electrophoretic mobility of Lrp-DNA complexes through native acrylamide gels of different porosities (Ferguson plots) (18). Fig. 1A shows the mobility versus percent acrylamide concentration for each of the standards used (globular proteins of molecular mass 14.2–132 kDa), and in Fig. 1B the slopes of each curve are plotted against the respective molecular mass. Lrp-DNA complexes, analyzed together with standards in the same gels, behaved like proteins having a molecular mass of 65 kDa. This result establishes the stoichiometry as $D_2P_2$ (molecular mass = 58 kDa) rather than $D_2P_4$ (molecular mass = 116 kDa). The fact that the experimentally determined molecular mass of the complex was higher than the calculated value (65 versus 58 kDa) may be due to differences in shape between the standards employed (globular proteins) and the sample (DNA-protein complex).

The conclusion that two Lrp monomers bind to a single site was confirmed by employing the strategy of Hope and Struhl (22). An Lrp derivative having 12 extra amino acids at the NH$_2$ terminus, including 6 His residues (6XHis-Lrp), shows DNA binding characteristics that are almost identical to the wild type (data not shown). By performing electrophoresis for 15 h, Lrp-DNA and 6XHis-Lrp-DNA complexes were clearly separated (Fig. 2, lanes 1 and 2). A mixture of homo- and heterodimers was created by mixing the wild type and 6XHis proteins, denaturing in 6 M guanidine and renaturing by dialysis. A band shift experiment performed with this mixture and DNA having a single Lrp binding site showed bands corresponding to complexes of DNA with wild type homodimer, 6XHis-Lrp homodimer, and wild type/6XHis-Lrp heterodimer (Fig. 2, lane 3). This is the expected pattern for binding of two Lrp monomers to a single binding site (22).

Lrp Binding as a Function of DNA Length—Primer Extension Analysis—An analysis of both natural and synthetic DNAs established the following as the consensus sequence for Lrp binding: YAGHAWATTWTDCTR, where $Y$ = A/T, $H$ = “not G,” W = A/T, D = “not C,” R = A/G (12). We synthesized a double-stranded 15 mer having a sequence that closely matched the consensus and observed no measurable binding of this DNA to Lrp (data not shown). The fact that this same sequence bound Lrp tightly when embedded within a 50-mer sequence (12) suggested that strong binding required, in addition to a 15-mer recognition sequence, DNA that flanked that recognition sequence. We used the procedure of Liu-Johnson et al. (20) to investigate the extent of flanking sequences that were required for tight binding. Single-stranded 50mers having an embedded sequence that was closely related to the consensus were subjected to primer extension in the presence of dideoxynucleotides, yielding a nested set of primer-extended products differing in length by one base. The identities of the constructs used in primer extension are shown in Fig. 3, D and E. Here and elsewhere in this report, F refers to the strand in Fig. 3E that is written 5' to 3' left to right and R refers to the complementary strand shown in Fig. 3D, written 3' to 5'. The products of primer extension were mixed with Lrp, and DNA complexed with Lrp was separated from free DNA by electrophoresis. After removing protein, samples of both were fractionated by electrophoresis through acrylamide gels under conditions of

![Fig. 2. Evidence suggesting that two Lrp monomers bind to a single site on DNA. Wild type (lane 1) and 6XHis-Lrp (lane 2) when complexed with ilvIH site 2 DNA have different electrophoretic mobilities. A mixture of the two proteins, after denaturation, renaturation, incubation with ilvIH site 2 DNA, and electrophoresis, show complexes corresponding to Lrp homodimer, 6XHis-Lrp homodimer, and heterodimer (lane 3; note band of intermediate mobility). Because electrophoresis was performed for 15 h, the free DNA ran off the gel.

![Fig. 1. Molecular mass estimate of an Lrp-DNA complex. A, relative electrophoretic mobility (R,) of the indicated macromolecules as a function of the acrylamide concentration within native gels. For chicken egg albumin and carbonic anhydrase, the major and fastest-moving isomers, respectively, were analyzed. B, the slope of each curve from panel A was plotted against the known molecular mass of each protein standard. The arrow and black rectangle denote the positioning of the Lrp-site 2 complex on this standard curve, based upon its curve shown in Panel A. The predicted molecular mass of the Lrp-site 2 complex from this experiment is 65 kDa.

![Graph showing molecular mass estimate of an Lrp-DNA complex.](http://www.jbc.org/)

![Graph showing slope versus molecular weight.](http://www.jbc.org/)
denaturation. Each of the two strands of the 50 mer were analyzed by primer extension, giving the results shown in Fig. 3, A and B. This method yields the binding constants of each of the primer-extended fragments (K$_n$) according to the equation:

$$K_n = \frac{C_n D_o}{C_o D_n}$$

where K$_{50}$ is the binding constant for the full length 50 mer, C$_n$ and D$_o$ are the band intensities of the complex and free DNA for each fragment, and C$_o$ and D$_{50}$ are the band intensities of the complex and free DNA for the 50 mer. 

A potential conclusion from these experiments is that given 15 bp or so of flanking double-stranded DNA on one side of the consensus, only a relatively few base pairs (from 0 to 3) are required on the other side of the consensus to get tight binding. However, another possibility has to be considered, namely that the single-stranded DNA portions of these primer-extended molecules affect binding to Lrp. As shown below, that turns out to be true. It makes a difference whether the single-stranded DNA is on the left or right of the double-stranded regions, and whether the polarity of the single-stranded DNA is 5' to 3' or 3' to 5'. These conclusions are derived from a comparison of the results described above with the results of additional primer extension experiments summarized in Fig. 4. In parts A and C, the 50-mer R strand served as template, and the primer was the complementary 15-mer consensus sequence. In parts B and D, the configuration was the same as for A and C, but the single-stranded tail of the template strand to the left of the consensus was not present. To facilitate a comparison of all of these results, we summarize them in Fig. 5 using a simplified notation. Binding of the double-stranded 50 mer to Lrp is set at 100% (Fig. 5, line 1), and other results are relative to this. Note that, for each of the comparisons in Fig. 5, the 15-bp consensus region is double-stranded. It is clear that single-stranded DNA flanking the consensus can significantly contribute to binding (Fig. 5, compare lines 2 and 4 with line 5). Furthermore, the polarity of the single-stranded DNA to the left of the consensus affects the degree of binding (Fig. 5, compare lines 2 and 4). Finally, the positioning of double-stranded and single-stranded DNA relative to the consensus affects binding (Fig. 5, compare lines 2 and 3). Thus, the construct having double-stranded DNA to the right of the consensus and single-stranded DNA to
FIG. 5. Summary of Lrp binding to DNAs having flanking double and single-stranded regions. Each DNA construct has a 15-bp double-stranded sequence that closely matches the consensus (shaded region), and flanking DNA that is either double-stranded or single-stranded. In each schematic, the top strand has polarity 5′ to 3′.

The left shows stronger binding than the construct with the opposite configuration.

Lrp Binding as a Function of DNA Length: Experiments with Oligonucleotides of Defined Length—A set of double-stranded DNA molecules were prepared having lengths 15, 17, 19, 21, 23, and 25 bp with the consensus occupying the center of molecules (Fig. 6A). Fig. 6, B and C, shows the relative binding of these constructs as measured by a gel retardation experiment. The 15, 17, and 19 mers showed almost no binding, whereas the 21, 23, and 25 mers showed substantial binding relative to the double-stranded 50 mer. Thus, 3 bp flanking each side of the consensus is sufficient for binding, and 5 bp flanking each side is nearly as good as a stretch as long as 25 bp.

In another set of experiments, we investigated the binding of Lrp to constructs having different lengths of single-stranded DNA flanking a constant double-stranded consensus region (Fig. 7, A and B). We chose 17 bp as the length of the double-stranded consensus region because such a minimal sequence did not bind Lrp (Fig. 6), and therefore we could expect to see an effect of single-stranded flanking DNA. In addition, a preliminary experiment showed that a 17:50 mer complex (and 19:50, 21:50, 23:50, and 25:50 complexes) bound Lrp with avidity equal to or greater than the double-stranded 50 mer (data not shown). As shown in Fig. 7C, if the flanking single-stranded DNA was of polarity F, then as little as two additional bases flanking each side of the double-stranded region contributed to increased binding and additional bases added to the strength of binding. The majority of added binding strength, however, was contributed by single-stranded DNA that was longer than 4 bases. The contribution of flanking single-stranded R strand to binding also was substantial only with a relatively long length of DNA, and was always less than that for an equivalent length of F DNA. These results are consistent with those mentioned above indicating that single-stranded DNA can contribute to Lrp binding and that the contribution of F and R single-stranded DNA is different.

The results in Figs. 6 and 7 demonstrating that both double- and single-stranded flanking DNA can contribute to Lrp binding were obtained in experiments employing only a single Lrp concentration. In a separate experiment employing just two of the constructs, we measured complex formation over a range of Lrp concentrations including Lrp in great excess. The apparent binding constants for Lrp binding to the double-stranded 50 mer and the 17R:50F construct were very similar (1.5 versus 1.7 nM, respectively), confirming the data for those constructs in Fig. 6.

The Effect of NaCl and Competitor DNA on Binding of Lrp to Constructs Having Flanking Double- or Single-stranded DNA—We measured the relative stability of Lrp-DNA complexes as a function of NaCl concentration. We chose the 50 mer F strand hybridized to a complementary 17 mer (Fig. 8A), a construct that binds Lrp with relatively high affinity (Fig. 7C). In parallel, we investigated the effect of salt on binding of Lrp to the double-stranded 50 mer. As shown in Fig. 8B, the effect of salt was larger on the construct with flanking single-stranded DNA than on the double-stranded construct.

In addition, we measured the effects of competitor DNA on binding of Lrp to the two constructs shown in Fig. 8A. The competitor DNAs were single and double-stranded forms of a 44 mer having a sequence unrelated to the Lrp binding consensus. Neither single nor double-stranded DNA competed very effectively against either of the constructs bound to Lrp: the binding constant was reduced less than two fold by competitors in 50–100-fold molar excess (data not shown).

DISCUSSION

For each of six operons thought to be directly controlled by Lrp, DNase I footprinting studies suggest that Lrp interacts with or affects the structure of DNA over a region of 100 bp or more (4, 6–10). Lrp clearly interacts with multiple sites within these regions, and in the cases of fimA (4), ilvH (5), lysU (6), and pap (3) those interactions are cooperative. Here we define the stoichiometry of interaction as two Lrp monomers binding

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A consensus for Lrp binding was formulated by a computer analysis of sequences associated with the Lrp regulon (23) and by a comparison of naturally occurring Lrp binding sites (11). A more extensive analysis of 63 binding sites derived by using the Selex approach of Tuerk and Gold (13) yielded a consensus sequence 15 bp in length that was palindromic in part and that was very similar to the consensus sequences derived previously (12). The work presented here indicates 15 bp may be sufficient for specificity, but an additional 3–5 flanking base pairs are required for tight binding. These additional flanking base pairs may contribute additional binding energy, as was proposed for the Crp/DNA interaction (20). Alternatively, DNA ends may be disruptive to binding, and strong binding may require that these ends be displaced from the site of protein-DNA contact by several base pairs. We cannot distinguish between these two possibilities.

Lrp binds tightly to double-stranded DNA having a sequence related to the consensus, but not to that same DNA after it has been denatured (5). Thus, Lrp does not bind tightly per se to single-stranded DNA. However, as demonstrated here, single-stranded DNA can stimulate Lrp binding when it flanks a double-stranded consensus sequence. There are at least three ways in which single-stranded flanking DNA could affect binding of Lrp. Single-stranded flanking DNA might bind to the same site on Lrp that normally binds double-stranded flanking DNA. The effects of NaCl on binding suggest that this possibility is not correct. Part of the binding of Lrp to DNA is likely driven by an increase in entropy due to the release of Na ions from the DNA (24). Binding of Lrp to double-stranded DNA is expected to release more sodium ions than binding to an equivalent length of single-stranded DNA and thus higher sodium ion concentrations should reduce binding to double-stranded DNA more than binding to single-stranded DNA. In fact, the opposite is the case (Fig. 8B). Another possibility is that single-stranded DNA interacts with Lrp at a second site and that this binding increases the strength with which the consensus sequence interacts with its binding site on Lrp. This possibility seems ruled out by the data showing that single-stranded competitor DNA reduces rather than increases binding to Lrp. A third possibility is that Lrp interacts only weakly with single-stranded DNA at some site other than the major binding site, but that this weak interaction provides the few kilocalories of energy required for binding of Lrp in the nanomolar range to the 17R:50F construct. The results of the competition experiment are consistent with this interpretation. The fact that single-stranded competitor DNA did not compete effectively can be explained by the fact that its concentration, although high relative to the labeled DNA, is much lower than the effective concentration of tethered single-stranded DNA.

Data summarized in Fig. 5 indicate that the binding strength conferred by single-stranded DNA depends upon whether that DNA is on the left or the right of the core binding sequence, and upon which of the two strands is single stranded. The difference in binding strength may reflect differences in the sequences of the single-stranded regions and/or differences in the halves of the 15-bp core binding sequence. The consensus sequence is partially palindromic (12) and for the particular sequence used here, one of the two half sites is calculated to be a better match to the consensus than the other (12). Thus, it is likely that one of the two subunits of Lrp is bound more tightly than the other, and this might contribute to the asymmetry observed in the binding of flanking single-stranded DNA.

It is not clear that the binding of single-stranded DNA to Lrp has any biological significance. However, one can imagine possible roles for such an activity (for example, binding to single-stranded DNA produced during transcription initiation), and
therefore it will be of interest to determine whether mutant Lrps having altered regulatory properties are also altered in their ability to bind single-stranded DNA.

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