An Lrp-like Transcriptional Regulator from the Archaeon
*Pyrococcus furiosus* Is Negatively Autoregulated*

Received for publication, July 6, 2000, and in revised form, August 21, 2000
Published, JBC Papers in Press, September 5, 2000, DOI 10.1074/jbc.M005916200

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The archaeal transcriptional initiation machinery closely resembles core elements of the eukaryal polymerase II system. However, apart from the established basal archaeal transcription system, little is known about the modulation of gene expression in archaea. At present, no obvious eukaryal-like transcriptional regulators have been identified in archaea. Instead, we have previously isolated an archaeal gene, the *Pyrococcus furiosus lrpA*, that potentially encodes a bacterial-like transcriptional regulator. In the present study, we have for the first time addressed the actual involvement of an archaeal Lrp homologue in transcription modulation. For that purpose, we have produced LrpA in *Escherichia coli*. In a cell-free *P. furiosus* transcription system we used wild-type and mutated *lrpA* promoter fragments to demonstrate that the purified LrpA negatively regulates its own transcription. In addition, gel retardation analyses revealed a single protein-DNA complex, in which LrpA appeared to be present in (at least) a tetrameric conformation. The location of the LrpA binding site was further identified by DNaseI and hydroxyl radical footprinting, indicating that LrpA binds to a 46-base pair sequence that overlaps the transcriptional start site of its own promoter. The molecular basis of the transcription inhibition by LrpA is discussed.

Recent studies have revealed that the archaeal transcriptional machinery represents a simplified version of the eukaryal RNA polymerase II transcription apparatus, which involves homologues of the TATA-binding protein (TBP),1 the transcription factor IIB (TFIIB; the archaeal homologue is called TFB), and the multi-subunit RNA polymerase II (for a recent review, see Ref. 1). The initiation process starts when the TBP interacts specifically with the core promoter element, the TATA box, which is located at positions −25 to −30 relative to the transcriptional start site (+1). This complex is stabilized by TFB, which interacts with TBP as well as with the nucleotides −42 to −19 that flank the TATA box (2). In particular, a sequence upstream of the TATA box (called the TFB-responsive element or BRE) is essential for transcriptional polarity (3, 4). Formation of this pre-initiation complex results in recruitment of the RNA polymerase complex (1). Although important progress has recently been made with the elucidation of the archaeal transcriptional mechanism, very little is yet known about the actual regulation of this process. A limited number of studies reported that expression of genes involved in nitrogen metabolism, methanogenesis, and sugar metabolism are subject to substrate-dependent regulation at the transcriptional level (5–9). Unfortunately, data about the molecular mechanisms underlying this regulation are still scarce. One of the few transcriptional regulators that have recently been studied in more detail concerns GvpE, an activator that is required for the expression of genes involved in gas vesicle synthesis in halophilic archaea. In a molecular modeling study, GvpE has been proposed to resemble a eukaryal leucine-zipper dimer that might interact with a palindromic sequence of its target promoter centered 40–50 bp upstream of the transcriptional start site (10). Another putative transcriptional regulator that has been studied in more detail is Tfx from *Methanobacterium thermoautotrophicum* (11). The tfx-encoding gene is located upstream of the operon encoding molybdenum formylmethanofuran dehydrogenase (*fmdECB*). Tfx binds to a site located 167 bp downstream of the transcriptional start site of *fmdE*. It was proposed that Tfx is a transcriptional activator required for the expression of *fmdECB*. Obvious homologues of Tfx can only be found within the domain of the archaea.

Analysis of the available archaeal genomic sequences shows that the majority of the identified homologues of regulators are bacterial-like (12). Recently a mechanism by which a bacterial-like regulator affects the archaeal transcriptional machinery was described. It was shown that MDR1 from *Archaeoglobus fulgidus*, a homologue of the iron-dependent bacterial repressor DxtR, represses transcription by binding to its own promoter in a metal-dependent manner. Upon binding of MDR1 to the promoter, RNA polymerase recruitment is prevented but not binding of TBP or TFB (13).

One particular group of bacterial-like regulators present in all available archaeal genomes is the family of Lrp/AsnC regulators. Members of this family have been identified in more than a dozen different bacterial species, in which they generally appear to be involved in regulation of amino acid metabolism. The most extensively studied example is the leucine-responsive regulatory protein (Lrp) from *Escherichia coli* (14, 38160 This paper is available on line at http://www.jbc.org
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15. Lrp is a global regulator that controls the expression of approximately 75 genes, many of which are involved in transport, degradation, or biosynthesis of amino acids. Lrp can either activate or repress transcription, and this action can be modulated by the effector leucine, which either decreases or increases its particular action. In some cases, like the negative autoregulation, leucine has no effect at all. The paralogous proteins binds to its promoter at a region overlapping the TATA box. In addition, it was shown that the *lrs14* transcript accumulates in the late growth stages of *S. solfataricus*.

In the genome sequence of *Pyrococcus furiosus* (Center of Marine Biotechnology, University of Utah) at least 10 homologues of genes encoding Lrp/AsnC-like proteins can be identified. The *lrpA* gene, encoding one of these homologues, was previously identified downstream of the *gdh* gene that encodes glutamate dehydrogenase (20, 21). In this paper, we describe the cloning, functional expression, and characterization of LrpA and show that LrpA binds to its own promoter and specifically inhibits transcription from this promoter. Using the combined data of gel mobility shift assays, in vitro transcription analyses, and footprinting, we identified the sequence elements responsible for LrpA binding and propose a mechanism by which LrpA binds its promoter.

### EXPERIMENTAL PROCEDURES

#### DNA Sequence Analysis—Identification of LrpA homologues was done using the Advanced BLAST program at NCBI. Alignments were made using the program ClustalX. Motif searches were performed using the PROSITE Pattern and Profile Searches program at the Expasy Molecular Biology Server and the program HELIX-TURN-HELIX (32). Inverted repeats were identified using the GeneQuest program, which is part of the DNA Star package.

#### Plasmid and Strain Construction—The gene encoding *lrpA* was PCR-amplified using primers BG240 and BG241 (see Table I, italic and underlined sequences indicate the restriction sites *Bsp*HI and *Bam*HI, respectively). The resulting 444-bp PCR fragment was cloned into pGEM-T (Promega Corp.), resulting in pLWU600, and the sequence of the insert was verified by DNA sequencing. Subsequently, pLWU600 was digested with *Bsp*HI and *Bam*HI, and the resulting 428-bp fragment was cloned into the T7 expression vectors pGEF (22), pET9d, and pET24d (23) (Novagen, Inc.), resulting in the constructs pLWU601, pLWU602, and pLWU605, respectively. The constructs were transformed into *E. coli* BL21(DE3), BL21(DE3) (pLas), and BL21(DE3) (pLyeE) (Novagen, Inc.) and tested for expression (not shown). The optimal result was obtained with *E. coli* BL21(DE3) in combination with the pET9d-derivative pLWU604. This combination was used for further expression experiments. pLWU613 was made by cloning fragment A into pGEM-T (Promega, Corp.). Mutations in fragment A were introduced using *Pfu* polymerase in the PCR-based overlap extension method (24). For each mutation a sense/antisense primer pair was designed. BG730 and BG731 introduced mutation 621; BG759 and BG760 introduced mutation 623; BG792 and BG793 introduced mutation 629; BG794 and BG795 introduced mutation 630 (see Table I). BG289 and BG290 were used as flanking primers for the PCR of fragment A (see below). All mutant fragments A were cloned into pGEM-T (Promega) and sequenced, resulting in pLWU621, pLWU623, pLWU629, and pLWU630.

#### Gel Mobility Shift Experiments—DNA probes used for gel mobility shift experiments were generated using PCR. The following primers were used: BG289 and BG290 for fragment A; BG367 and BG290 for fragment B; BG289 and BG431 for fragment C; BG430 and BG290 for fragment D; BG289 and BG431 for fragment E; and BG289 and BG615 for fragment F. These constructs were used as a template in PCR reactions with a *Pfu* polymerase (Stratagene), and clones were selected containing the insert in both orientations.

| Name | Sequence (5’ → 3’) |
|------|--------------------|
| BG240 | TTATTACAGGCGTATAGTTT |
| BG241 | GCCGGGATCTTCTAGTTTCAGGACAAATATATA |
| BG289 | CAGGAGAATTCGTTGACCAATCCGGAAA |
| BG290 | GGATGAGACGCAAACAGTGGT |
| BG427 | CTCTAGAATCAGCACTAGAATATAAAGCTATGCCCC |
| BG430 | TTACTTAAGTTTTTCAAGGATTATAGTCATGA |
| BG431 | CTCTAGAATGTTCAACACTATGGCTC |
| BG498 | CATCAATCATTTTTCGAACCACCTAGGTATAAC |
| BG615 | BG638 and BG498 for fragment E; BG638 and BG615 for fragment B; BG289 and BG431 for fragment C; BG430 and BG290 for fragment D; |
| BG636 | TGTGCTGATTTATATATATCTTA |
| BG730 | CCTGATTTATAAATATGTTTCGAAAA |
| BG731 | TTTTGGGACATTTATTTAATAACCATGCACG |
| BG759 | TTTTGTCTGAGGTTGCTTATCAGGACATCCGGAAA |
| BG760 | TTTTTCGAACCACCTAGGTATAAC |
| BG792 | TTTTTCGAACCACCTAGGTATAAC |
| BG793 | TTTTGGGACATTTATTTAATAACCATGCACG |
| BG794 | AATACCTAGTTTTAGGAAAAATGATTGATGAG |
| BG795 | AATACCTAGTTTTAGGAAAAATGATTGATGAG |

#### Overproduction of LrpA—The *P. furiosus* LrpA protein was produced in 2-liter Erlenmeyer flasks containing 1 liter of LB medium with 50 μg/ml kanamycin. The culture was inoculated with *E. coli* BL21(DE3) transformed with pLWU604. Cells were grown in a rotary shaker at 37 °C until an *A*<sub>600</sub> of 0.5 was reached, and 0.4 ml isopropyl-1-thio-β-n-galactopyranoside was added to induce expression. After overnight incubation, the cells were harvested, washed in 125 mM citrate buffer, pH 5.0 and resuspended in 90 ml of the same buffer. Cells were lysed by a triple passage through a French pressure cell at 1000 p.s.i. After lysis, MgCl<sub>2</sub> and DNaseI were added to final concentrations of 10 mM and 10 μg/ml, respectively. The sample was left at room temperature for 15 min. Subsequently, the cell-free extract was incubated at 80 °C for 30 min and centrifuged at 20,000 rpm for 30 min. The remaining soluble fraction was loaded on a 60-ml cation exchange column (S-Sepharose, Amersham Pharmacia Biotech) that had been equilibrated with 125 mM citrate buffer, pH 5.0. The column was eluted with the same buffer using a flow rate of 3 ml/min and a linear gradient of NaCl from 0 to 1 M. Fractions containing LrpA, as determined by SDS-PAGE, were pooled and concentrated by centrifugation in Centricon units (10-kDa cutoff) until a volume of 0.5 ml was reached. A 200-μl sample was loaded on a gel filtration column (Superdex 200, Amersham Pharmacia Biotech) with 20 mM Tris, pH 8.0, and 100 mM NaCl with a flow rate of 0.5 ml/min. The elution pattern from this gel filtration showed three peaks corresponding to molecular masses of approximately 30, 69, and 120 kDa, respectively. Approximately 14 ng of purified LrpA was obtained from one liter of culture.

#### Gel Mobility Shift Experiments—DNA probes used for gel mobility shift experiments were generated using PCR. The following primers were used: BG289 and BG290 for fragment A; BG367 and BG290 for fragment B; BG289 and BG431 for fragment C; BG430 and BG290 for fragment D; BG638 and BG431 for fragment E; and BG289 and BG615 for fragment F. The constructs were used as a template in PCR reactions with a *Pfu* polymerase, and clones were selected containing the insert in both orientations. Each reaction consisted of 5-μl final extension step at 75 °C, 30 cycles consisting of 95, 45, and 72 °C, with 30 s for each step, followed by a 7-min final extension step at 72 °C. PCR products were end-labeled using T4 kinase and radioactive [γ-<sup>32</sup>P]ATP. Binding reactions were performed in a total volume of 20 μl containing 40 mM HEPES–NaOH pH 7.3, 200 mM KCl, 2.5 mM MgCl<sub>2</sub>, 2 mM dithiothreitol, 1 mM CaCl<sub>2</sub>, 100 mM EDTA, 10% glycerol, and varying concentrations of purified LrpA. Standard reactions contained 2 μg of poly(dI-dC)·poly(dI-dC) as nonspecific competitor DNA, but this was omitted from reactions with smaller fragments (fragment E and F) and during determination of the dissociation constant (K<sub>d</sub>) for the LrpA-DNA complex. Each reaction contained 1 to 10 ng of specific competitor DNA. Reactions were incubated at room temperature for at least 10 min and separated on a non-denaturing 8% acrylamide gel buffered in 1× Tris borate EDTA buffer (25). In the case of fragment F, a 20% gel was used. Gels were dried, exposed to phosphor screens, and analyzed. Quantification was done using ImageQuant software (Molecular Dynamics, Inc.).
DNaseI reaction was stopped by the addition of 20 mM loading buffer, heated at 95 °C for 5 min, and chilled on ice. Subsequently, 0.8 μl was analyzed on a Li-Cor 4000 sequencer using a 5.5% RB 

denaturating sequence gel (Li-Cor) with 0.2-mm spacers and settings 2000 V, 25 mA, 50 watt, and 45 °C.

Hydroxyl radicals were generated by adding 3 μl of 40 mM sodium ascorbate, 3 μl of 1.2% H2O2, and 3 μl of 4 mM (NH4)2SO4 in H2O, 8 mM EDTA. After 2 min the reaction was stopped by the addition of 26 μl of 0.1 M thioxanthone, 20 μl EDTA. DNA was extracted with phenol, precipitated as described above, and analyzed on a Li-Cor 4000L sequencer (Li-Cor) using a 6-6 cm denaturing sequencing gel with 0.2-mm spacers and settings 2000 V, 25 mA, 50 watt, and 45 °C. Images of the footprints were analyzed using the program Scion Image for Windows, available from the National Institutes of Health.

In Vitro Transcription—Transcription reactions were performed essentially as described previously (26) except that 300 mM KCl was used instead of 250 mM. A standard reaction mixture (50 μl) contained 1 μg of linearized template DNA (pLUW479, 28), pLUW613, pLUW621, pLUW623, pLUW629, pLUW629, or pLUW512 (27), 250 ng of recombinant TBP, 135 ng of native RNA polymerase, and varying concentrations of LrpA. This reaction mixture was incubated for 30 min at 37 °C. RNA purification and electrophoresis was performed as described previously (28).

Primer Extension—For analysis of the in vitro transcriptional start site, cell-free transcription reactions were performed as described above but without labeled precursors. In a control reaction, nucleotides were omitted from cell-free transcription reactions. The end-labeled DNA primer 5'-GTATAATTGTTGCTCCTCTCATCA-3' was used complementarily to nucleotides +20 to +42 relative to the transcriptional start of lrpA. The primer extension assay was performed as described previously (26, 28). For determination of the in vivo transcriptional start site, primer extension was performed with total RNA of P. furiosus, which was isolated as described previously (29).

Chemical Cross-linking and Western Blot Analysis—Chemical cross-linking was performed as described by Davies and Stark (30), with the following modifications. For cross-linking experiments with free LrpA, different concentrations of LrpA were diluted in cross-linking buffer (80 mM trithiocarbonamide-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.1 mM dithiothreitol), and the final volume was adjusted to 16 μl. Dimethyl-suberimidate (DMST, 25 mM freshly made in cross-linking buffer) was added to a final concentration of 5 mM so that the final volume was 20 μl. After a 1-h incubation at room temperature, SDS-PAGE loading buffer (25) was added, and 100 ng of each sample was separated on a 10% Tricine SDS-PAGE gel (31). The separated proteins were transferred to a nitrocellulose membrane by electrophoretogating in 10 mM CAPS, pH 11.0, and 10% methanol and detected immunoologically using a polyclonal antiserum raised against purified LrpA (25). For cross-linking experiments with LrpA-DNA complexes, about 2 μg of purified LrpA was incubated with 500 ng of DNA (fragment B, see Fig. 1B) in cross-linking buffer in a final volume of 64 μl. DMST was added as described above so that the final volume was 80 μl. The samples were loaded on a non-denaturing 5% acrylamide gel, buffered in 1× Tris borate EDTA buffer (25). The gel was stained with ethidium bromide, bands representing specific DNA-LrpA complexes were excised and crushed, and SDS-PAGE loading buffer was added. The recovered samples were heated for 10 min at 100 °C and loaded on a 10% Tricine SDS-PAGE gel and analyzed as described above.

RESULTS
LrpA Sequence Analysis—A 2.7-kilobase HindIII fragment including the gene encoding glutamate dehydrogenase (gdh) was previously isolated from a genomic library of P. furiosus (Fig. 1R) (20). Downstream of the gdh gene, an open reading frame was found with a high degree of similarity to bacterial transcription regulators of the Lrp/AsnC family (21).

Analysis of the lrpA gene identified a frame-shift in the previously published sequence (GenBank TM accession number P42180), which introduced a stop codon after lysine 120 in the predicted protein sequence. The corrected P. furiosus lrpA gene is predicted to encode a 141-amino acid protein with a predicted molecular mass of 15.9 kDa. Subsequent BLAST analysis revealed that LrpA shares a high degree of similarity with many (hypothetical) regulatory proteins from a number of archaea including Pyrococcus horikoshii (93% identity), Pyrococcus abysii (98% identity), Methanococcus jannaschii (54% identity), A. fulgidus (49% identity), M. thermoautotrophicum (57% identity), and S. solfatarius (32% identity, Fig. 2). In addition, all of these archaea contain a number of more distantly related homologues, e.g., in P. horikoshii a total of 9 genes appear to encode LrpA homologues, whereas P. furiosus itself contains at least 10 LrpA homologues. The best characterized LrpA homologues are from bacterial origin, in particular E. coli AsnC (33% identity) and E. coli Lrp (28% identity). A PROSITE pattern search with P. furiosus LrpA identified a putative helix-turn-helix motif of the Lrp/AsnC family (Fig. 2). This motif was also predicted by the program HELIX-TURN-HEXIL (32).

Overexpression and Purification of LrpA—Using a PCR approach we cloned the lrpA gene into a PET9d vector, resulting in pLUV604. Production of LrpA was achieved after transformation of pLUV604 into E. coli BL21(ADE3). After overnight growth in the presence of 0.4 mM isopropyl-1-thio-β-D-galactopyranoside, cells were harvested and disrupted. Tricine-SDS-PAGE analysis of membrane and soluble fractions indicated that 50% of the produced LrpA was present as soluble protein (Figs. 3, lane 3, and 4). The soluble fraction containing LrpA was further subjected to a heat incubation of 30 min at 80 °C, resulting in the denaturation of most of the E. coli proteins (Fig. 3, lane 5). This heat-stable cell free extract was used for further purification by cation exchange chromatography and gel filtration chromatography (Fig. 3, lanes 6 and 7). The calculated molecular mass of LrpA is 15.9 kDa, which is in good agreement with its migration on SDS-PAGE (Fig. 3). Elution patterns from gel filtration showed peaks corresponding to
molecular masses of approximately 30, 60, and 120 kDa. This suggests that LrpA exists as a dimer, tetramer, and octamer in solution. We performed several independent gel filtration experiments with LrpA, and the apparent oligomeric heterogeneity was always observed.

Analysis of the lrpA Promoter—We used primer extension analysis to map the transcriptional start site for lrpA. Using in vitro generated run-off transcript RNA (see below), we found that the transcriptional start was located at an adenosine located 14 bp upstream the translational start (see Fig. 1A). We compared the lrpA promoter sequence to other known promoter sequences from P. furiosus. Although only 14 Pyrococcus promoters have been mapped to date, a clear consensus sequence can be derived for the Pyrococcus BRE and TATA elements: AAAnnTTTWWWWW (−35 to −23 sequence relative to the transcriptional start (+1), where n = any base, and W = A or T). The putative BRE and TATA elements of the lrpA promoter match well with the consensus sequence mentioned above (see Fig. 1A).

We used total isolated RNA from P. furiosus grown on cellobiose, pyruvate, and tryptone to determine the transcriptional start in vivo. In all cases the transcriptional start was identical to that found with in vitro generated RNA (not shown); however, relatively weak signals were obtained. Although the results showed that lrpA is expressed during growth on the above-mentioned substrates, they indicate that lrpA transcript levels are not very abundant under these conditions.

In Vitro Transcription of lrpA and gdh in the Presence of LrpA—Several bacterial members of the Lrp/AsnC family are negatively autoregulated (16, 33, 34). Hence, it was anticipated that P. furiosus LrpA could also be a repressor of its own expression. In the previously established cell-free transcription system (26), the P. furiosus purified transcription factors TBP, TFB, and RNA polymerase direct efficient transcription from a
DNA template containing the (partial) gdh-encoding sequence and its promoter. We used this in vitro transcription system to study the effect of LrpA on lrpA and gdh transcription (Fig. 5). The template used in this experiment is PstI-linearized pLUW613, which carried fragment A (Fig. 1B). Transcription from this template results in a 160-nucleotide lrpA run-off transcript. With increasing amounts of LrpA present in the reactions, the signal of the radiolabeled lrpA PstI run-off transcripts drastically decreased (Fig. 5A). This indicated that LrpA had a negative effect on its own transcription in vitro. A similar experiment was performed using BamHI-digested pLUW479 as the DNA template (26). This plasmid carried the partial P. furiosus gdh gene including its 200-bp upstream sequence. Transcription from this template resulted in a 173-nucleotide gdh run-off transcript. There was no effect on gdh transcription when LrpA was added (Fig. 5B). Likewise, transcription from the Methanococcus vanNielii tRNAval promoter (26) was not inhibited by LrpA (Fig. 5B).

LrpA Binds Specifically to Its Own Promoter—To test whether LrpA binds to its own promoter, we performed gel mobility shift experiments with purified LrpA and DNA fragments containing the LrpA promoter sequence. Three different DNA fragments (C, D, and E) were used that contained sequences upstream of lrpA (Fig. 1B). Fragment C contained the upstream lrpA promoter region including the TATA element (Fig. 1B). LrpA did not shift this fragment, indicating that no interaction occurred between LrpA and the upstream promoter region (Fig. 6). Fragment D contained the TATA element, the transcriptional start site, and the sequence downstream thereof (Fig. 1B). The addition of LrpA to this fragment resulted in a shifted band (Fig. 6). Using LrpA concentrations of up to $8 \mu$g (as calculated for an LrpA monomer), we observed only one shifted band in gel mobility shift experiments, indicating that only one complex is formed when LrpA binds to the lrpA promoter. This binding was specific, since the addition of 2 $\mu$g of poly(dI-dC)-poly(dI-dC) as nonspecific competitor DNA did not prevent LrpA-DNA binding.

As mentioned above, we observed dimer, tetramer, and octamer configurations of LrpA in gel filtration chromatography experiments. We tested fractions of all these different forms in gel mobility shift experiments; however, there was no obvious difference in DNA binding activity between the fractions (not shown).

To determine the affinity of LrpA for its promoter DNA, we performed a gel mobility shift experiment with fragment D and increasing LrpA concentrations (not shown). The concentration of LrpA, as calculated for an LrpA monomer, that caused half of the DNA to become complexed under the experimental conditions used was taken as the dissociation constant ($K_D$) of the LrpA-DNA complex (35). We determined a $K_D$ of 0.3 nM, which is about 4–10-fold lower compared with values measured for E. coli Lrp (36, 37) and several orders of magnitude lower than that of S. solfataricus Lrs14 (19).

We tested several smaller fragments to locate the boundaries of the LrpA binding sequence more precisely. Fragment E (43 bp) was the smallest fragment to which LrpA bound efficiently (Figs. 6 and 7C). LrpA also bound to fragments smaller than 43 bp, such as fragment F (30 bp), but the affinity for these fragments was drastically decreased (Fig. 6 and 7C). LrpA binding to fragment E was specific, since the addition of increasing amounts of unlabeled fragment E prevented LrpA binding to labeled fragment D (not shown). Altogether, these results indicated that LrpA binds specifically to its own promoter at a position around the transcriptional start site. Since P. furiosus grows optimally at 100 °C we performed binding reactions for gel mobility experiments at several temperatures. Binding experiments performed at 0, 4, 25, 50, and 80 °C resulted in identical gel mobility shift patterns (not shown).

Gel mobility shift experiments were also performed with DNA fragments containing gdh promoter sequences. Under similar conditions as the experiments with the lrpA promoter fragments, we observed very weak LrpA binding with fragment G (not shown). This fragment contained 386 bp of the gdh promoter, including the TATA element (Fig. 1B). However, this binding appeared to be rather weak and nonspecific since the observed shift disappeared upon the addition of poly(dIdC)-poly(dIdC). This indicates that, under the tested conditions, LrpA binds specifically to its own promoter but not to the gdh promoter.

DNases I and Hydroxyl Radical Footprinting—To study LrpA binding at its own promoter in more detail, we performed
DNaseI footprinting with purified LrpA and fragment B containing the lrpA promoter (Fig. 1B). The addition of LrpA resulted in DNaseI protection at a region of −19 to +21 relative to the lrpA transcriptional start (+1, Fig. 7A). This indicated that this region contained the LrpA binding sequence. Additionally, sites hypersensitive to DNaseI cleavage were observed. Such an effect is common when DNA-binding proteins bind to their target DNA and can be explained as protein-induced DNA bending (38). In the case of LrpA, however, apparent hypersensitivity appeared mainly at positions outside of the −19 to +21 region, suggesting that binding of LrpA affected the DNA conformation upstream and downstream of its binding site (see “Discussion”). DNaseI footprinting experiments were also performed with purified LrpA and fragment G containing the gdh promoter. As in gel mobility shift experiments, no interaction between LrpA and gdh promoter DNA could be detected (not shown).

To further characterize the LrpA binding site(s) at its own promoter, hydroxyl radical footprinting was performed with the lrpA promoter fragment B (Fig. 1B). Using this technique information can be obtained about the interactions between LrpA and the DNA sugar-phosphate backbone. The plot profiles of the relative band intensities from these experiments revealed the presence of four regions with decreased band intensities in both the non-template and the template strand (Fig. 7B). These regions are all located between positions −22 and +24. A spacing of approximately 10 bp was present between disappearing bands, which indicated that interactions took place along the same face of the double helix. A schematic summary of the data obtained from gel mobility shifts, DNaseI, and hydroxyl radical footprinting is given in Fig. 7C.

**Mutational Analysis of the LrpA Binding Sequence**—Because most of the protection against DNaseI cleavage occurred within the −19 to +11 region, we tested whether LrpA was able to shift a 30-bp fragment (F, Fig. 7C) containing only this −19 to +11 sequence. This fragment is only partly shifted, even at higher LrpA concentrations (Fig. 6). Although binding to this fragment was, thus, much weaker than to larger fragments like E or D, sequence elements specifically recognized by LrpA are present within this region. Therefore, we designed mutations in this particular region for a more detailed analysis of the sequence elements required for the LrpA-DNA interaction. The effects of these mutations were tested in gel mobility shift experiments and in the cell-free transcription system mentioned above. In gel mobility experiments we used mutants of fragment B (Fig. 1B), and for the cell-free transcription experiments, we cloned mutants of fragment A (Fig. 1B) into pGEM-T. Although transcripational activities or transcriptional start sites were slightly altered for some mutant promoters, it was possible to study the effect of LrpA on transcription from the mutated templates.

We used different approaches to design mutations. In the first approach we mutated four of the eight bp of the palindromic sequence ACCTAGGT present within the −19 to +11 sequence (Fig. 6A, 612). In a gel mobility shift experiment, however, a DNA fragment containing this mutation shifted with an efficiency almost identical to that of the wild-type DNA fragment (Fig. 6B). It was impossible, however, to analyze the effect of this mutation by in vitro transcription, since no transcript was formed using this mutant DNA as a template. A sequence element crucial for transcription is apparently disturbed in this mutant. Most likely this element corresponds to (part of) the initiator element (INR, previously referred to as Box B) (39, 40), since it is located around the transcriptional start site.

In the second approach we compared putative lrpA promoter sequences of P. horikoshii (41) and P. abyssi with that of P. furiosus. All these Pyrococcus species have a similar organization of gdh and lrpA genes, and putative promoter sequences of lrpA share a high degree of homology. In Fig. 5A, the bases that are conserved among the three Pyrococcus species are indicated with a gray background. Upstream from the TATA boxes there is little conservation, whereas a higher degree of conservation is present around the TATA elements and the sequence downstream thereof. We mutated two conserved sequence blocks present within the −19 to +11 region (Fig. 5A, 623 and 629). When we substituted TTATAC into GGCGCA (623) we found that the affinity of LrpA for this mutant DNA was in fact higher than for the wild-type DNA sequence. In accordance, LrpA inhibited in vitro transcription more efficiently (125%, Fig. 8D). When we substituted TAGTGTGGT into GCTGTGTG TGG (629), there was no binding of LrpA in a gel mobility shift experiment. This was in agreement with the affected transcription inhibition, which was only 52% compared with wild-type DNA (Fig. 8B).

Finally, we focused on the right half of the substituted bases in promoter mutant 629, since bases at the left half did not cause a drastic effect on LrpA binding (mutant 621). Therefore, we constructed promoter mutant 630, in which GGTTC is substituted by TTGGA (Fig. 8A). This substitution caused the same effect as in mutant 629. LrpA did not bind the mutant promoter DNA, and in agreement with this, transcription inhibition by LrpA was decreased to 44% compared with wild-type DNA (Fig. 8B). The observation that there was still an effect in in vitro transcription, although there was no detectable binding to mutant fragments 629 and 630, can be explained by the fact that nonspecific competitor DNA was only added in gel mobility shift experiments. In the absence of competitor DNA, very weak LrpA binding still occurred (not shown).

**Chemical Cross-linking**—Results from gel filtration chromatography suggested that LrpA existed as a dimer, tetramer, and octamer in solution. To further characterize the configuration of LrpA as a free protein or in complex with lrpA promoter DNA, we performed chemical cross-linking experiments. DMSI was used as a cross-linking agent that acts by forming covalent amide linkages between lysine residues. This results in cross-
links primarily between the subunits of oligomeric proteins (30). Cross-linked LrpA was analyzed by SDS-PAGE, followed by Western blotting using an antiserum raised against purified LrpA. First, cross-linking experiments were performed with LrpA as a free protein in solution. For that purpose, LrpA was incubated with DMSI, and cross-linking was analyzed using SDS-PAGE. DMSI caused the appearance of three bands in addition to the LrpA monomer (I), corresponding to molecular masses of an LrpA dimer (II), trimer (III) and tetramer (IV). Although the band corresponding with the dimeric form (II) appears to be the predominant species, a band corresponding with the tetrameric form (IV) is present as well.

**Fig. 7.** A, DNaseI footprint analysis using purified LrpA and lrpA promoter DNA. 0, 30, 90, 270, 810, 2430, and 0 nM purified LrpA was incubated with labeled fragment B (see Fig. 1B). After DNaseI treatment, the DNA was analyzed in parallel with a sequencing reaction. Left, 5'-labeled non-template strand; right, 5'-labeled template strand. Brackets indicate DNaseI protection, and arrows refer to hypersensitive sites. B, profile plots of hydroxyl radical footprinting. Panel I, non-template strand; panel II, template strand. Nucleotide positions are given relative to the lrpA transcriptional start (+1). Upper traces, no LrpA added; lower traces (bold), 2.4 μM LrpA added, as calculated for monomeric protein. Profile plots at the bottom indicate sequence reactions that were run in parallel with hydroxyl radical cleavage reactions. Brackets indicate protection against hydroxyl radical cleavage in the presence of LrpA. C, schematic summary of results obtained from gel mobility shift experiments, DNaseI footprinting, and hydroxyl radical footprinting. A double-helical representation of the lrpA promoter region is given along the sequence of the non-template strand. Filled circles, protection against hydroxyl radical cleavage; filled arrows, protection against DNaseI cleavage; open arrows, DNaseI hypersensitive sites. Sequence positions are relative to the lrpA transcriptional start (+1). Boxed sequences indicate the BRE element, TATA element, and ATG start codon, respectively.

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**Links: Primary**

1. DMSI treatment
2. Western blotting
3. Cross-linking experiments
4. SDS-PAGE
5. Hydroxyl radical footprinting
6. DNaseI footprinting
7. Gel mobility shift experiments
8. Sequence analysis
9. Proteins interaction analysis

**Methods:**

- DMSI treatment
- Western blotting
- Cross-linking experiments
- SDS-PAGE
- Hydroxyl radical footprinting
- DNaseI footprinting
- Gel mobility shift experiments
- Sequence analysis

**Results:**

- Appearance of three bands
- DMSI causes the appearance of bands
- DMSI causes protection against cleavage
- DMSI causes protection against DNaseI cleavage
- DMSI causes hypersensitive sites

**Conclusion:**

- DMSI treatment of LrpA results in the appearance of bands corresponding to different oligomeric forms.
- Protection against hydroxyl radical and DNaseI cleavage is observed.
We tested several concentrations of LrpA (200 nM to 40 μM) under identical cross-linking conditions but did not observe an obvious change in the intensities of the individual bands (not shown). This suggests that the degree of multimerization of LrpA is not concentration-dependent under the tested conditions.

We also performed cross-linking experiments with LrpA bound to its target DNA. LrpA was incubated with lrpA promoter DNA, DMSI was added, and the samples were separated on a non-denaturing acrylamide gel. Subsequently the bands representing specific LrpA-DNA complexes were excised and analyzed by SDS-PAGE. The pattern of cross-linked LrpA in complex with DNA was almost similar to that of free LrpA (Fig. 9). In both cases bands with molecular masses corresponding to the dimer, trimer and tetramer or LrpA were present in addition to the monomer. However, in cross-linked LrpA that is complexed with DNA the tetrameric form appeared to be the more dominant species. Therefore, these results suggest that at least four LrpA monomers are in complex with the lrpA promoter fragment.

**DISCUSSION**

The present study describes the characterization of the LrpA transcriptional regulator from the hyperthermophilic archaeon *P. furiosus*. Comparison of the amino acid sequence of LrpA reveals that it belongs to the Lrp/AsnC family of transcriptional regulators, which consists of many bacterial proteins as well as a growing number of putative archaeal proteins (Fig. 2). Although gel filtration experiments showed that the purified recombinant LrpA exists as a mixture of dimer, tetramer, and octamer in solution, chemical cross-linking experiments suggest that the tetrameric form of the protein was the highest quaternary structure, both in solution and in complex with DNA (Fig. 9). In comparison, *E. coli* Lrp exists as a dimer, both in solution and in complex with a single binding site. *Pseudomonas putida* BkdR exists as a tetramer in solution, and three tetramers are proposed to bind to its target DNA (42–45).

In vitro analyses revealed that *P. furiosus* LrpA binds to its own promoter and represses its transcription. We tested potential effectors for their ability to alter the effect of LrpA on lrpA transcription; however, both in vitro transcription analyses and gel mobility shift assays revealed that these compounds had no effect on the LrpA autoregulation efficiency. Negative autoregulation is a common characteristic within the Lrp/AsnC family of regulatory proteins, and in general this repression is independent of effectors (16, 34, 46).

It has been reported that the RNA polymerase of the archaeon *Methanococcus thermolithotrophicus* protects a region of −30 to +20 at a number of promoters in DNasel footprinting experiments (47). In a similar approach, Hausner et al. (2) demonstrate that TBP and TFB together protect the −42 to −19 region of the *P. furiosus* gdh promoter. Assuming that these proteins bind the same region at the lrpA promoter, LrpA binding occurs immediately downstream of the bound TBP-TFB complex (−22 to +24, Fig. 7C). In this way, LrpA blocks at least the binding of the RNA polymerase, thereby inhibiting completion of the archaeal transcription initiation complex. Such a mechanism of repression has recently been demonstrated for the *A. fulgidus* metal-dependent transcriptional repressor MDR1 (13). Repression occurs when MDR1 binds to the −18 to +67 sequence of its target promoter, thereby preventing RNA polymerase recruitment but not binding of the TBP-TFB complex. Since the 5′ border of the LrpA binding sequence (−24) is comparable with that of MDR1 (−18), a similar mechanism of repression is anticipated.

In analogy with *E. coli* Lrp, it is tempting to speculate that the archaeal homologues may also act as global regulators (18), although the results from gel filtration chromatography showed that LrpA also exists as an octamer in solution, this configuration was not observed in cross-linking experiments.
and thereby act both as transcriptional repressors and activators. Activation of transcription would probably require binding upstream of the −42 to −19 region involved in binding of the TBP-TFB complex, as has been suggested for the Haloferax GvpE transcriptional activator (10). In addition, a probable requirement would be a specific interaction between the bacterial-like LrpA protein and one or more components of the eukaryal-like (pre-initiation complex. Although many genes encoding Lrp/AsnC homologues can be found within archaea, their role as transcriptional global regulators or activators remains speculative.

Hydroxyl radical footprinting analysis confirmed that LrpA protects its promoter from position −22 to +24 (Fig. 7B). We compared the size of the protected sequence (46 bp) to hydroxyl radical footprints obtained from E. coli Lrp in complex with ilvIH and ilvGMEDA (36, 37). In these cases two E. coli Lrp dimers bind to a dual binding site of about 45 bp. In addition, it has been shown that one Lrp dimer binds to a single binding site of about 15 bp (43). It is not unlikely that the P. furiosus LrpA binding sequence consists of two adjacent binding sites. In gel mobility shift experiments only one distinct LrpA-DNA complex is present. This suggests either binding of an LrpA tetramer or the highly co-operative binding of two LrpA dimers. In both cases four LrpA monomers are in complex with DNA, as was detected using chemical cross-linking of LrpA in a specific LrpA-DNA complex. Both of the above mentioned configurations (tetramer or two dimers) would explain why a fragment of at least 43 bp is necessary for a stable LrpA-DNA complex in gel mobility shift experiments (see Fig. 6). In contrast, for a stable interaction between an E. coli Lrp dimer and a single binding site, a DNA fragment of only 21 bp is necessary (43).

The 46-bp binding sequence has been analyzed for sequence motifs that could serve as targets for DNA-binding proteins (e.g. palindromes, inverted or direct repeats). An obvious 8-bp palindrome ACCTAGGT is present (Fig. 8A), but the possibility that this is a specific recognition element for LrpA can be ruled out because LrpA binds well to promoter mutant 621, in which the palindrome has partly been substituted (Fig. 8B). Moreover, the consensus binding sequence for E. coli Lrp consists of at least 15 bp. Assuming that LrpA binds to two sites, it might be expected that the two sites share similar sequence elements. Overall, however, there is very little sequence homology between the two halves of the 46-bp fragment. Although a CTAG motif is present in the left part of both of these halves (position −20 and −2), the combined results from our mutational analysis suggest that the GGTTC sequence and not the CTAG motif is present in the left part of both of these halves (position 20 and 22) (Fig. 6). This suggests that LrpA does not recognize a limited number of specific bases but rather a relatively long sequence with a specific secondary structure. Indeed, certain protein-DNA interactions have been reported to be the result of a specific DNA structure and/or flexibility (48). In addition, it should be noted that a consensus sequence for E. coli Lrp is in many cases not obvious at all.

The appearance of DNaseI hypersensitive sites in the proximity of DNaseI-protected regions is a very common phenomenon when DNA-binding proteins interact with their target DNA and is generally interpreted as a result of DNA bending. In some cases this bending occurs when DNA is looped out through interaction between proteins bound to sites spaced (far) apart (38). In many other cases, binding of a protein to a single binding site causes bending of DNA (49–51). DNaseI footprinting studies with these proteins, however, revealed only a small number of hypersensitive sites, generally located within the area protected against cleavage (2, 52–62). DNaseI footprints of E. coli Lrp with several target promoter fragments show protection from DNaseI over a range of 100 bp or even longer, due to multiple binding sites. Hypersensitive sites are located between the protected regions (37, 63–67). Similar patterns are generated by the Lrp homologs BkdR from P. putida (44) and PutR from Agrobacterium tumefaciens (46). In the case of P. furiosus LrpA, however, two distinct footprinting techniques indicate only a significant protection of a 46-bp fragment of the LrpA promoter, whereas DNaseI-hypersensitive sites are extended in both directions, in total covering approximately 88 bp (Fig. 7). Hence, at least under the conditions used for the DNaseI footprinting (elevated LrpA concentrations), LrpA appears to affect the DNA conformation over a relatively long distance. In the case of E. coli Lrp and A. tumefaciens PutR, it has been suggested that DNA is somehow wrapped around the regulators (66, 68). Analysis of PutR-DNA complexes by atomic force microscopy supports the idea that PutR condenses DNA into globular nucleoprotein complexes (46). Although a similar looping or wrapping of DNA around P. furiosus LrpA is possible, we cannot rule out the possibility that the observed hypersensitivity pattern is a result of some nonspecific phenomenon in the in vitro analysis.

In conclusion, we have described for the first time the actual involvement of an archaeal Lrp homolog in transcription modulation by in vitro analyses. The P. furiosus LrpA interacts specifically with its promoter in the proximity of the transcriptional start site. Hence, the observed transcription inhibition is most likely a consequence of preventing RNA polymerase recruitment similar to that reported for the A. fulgidus MDR1 (13). In addition, LrpA binds to lrpA promoter fragments in a single configuration, most likely as a tetramer. Alternatively, such a configuration may also be referred to as a dimer of dimers, but we do not have any indication of cooperativity, as has been reported for E. coli Lrp (Calvo et al. (14) and Newman and Lin (15)). Gel retardation analysis revealed that the DNA binding efficiency of LrpA is reduced significantly when the DNA fragments were reduced in size below 46 bp. The interaction with this fragment was confirmed in two distinct footprinting experiments. The actual binding site could at least in part be identified, but as in many bacterial Lrp-target promoters, no obvious palindromic motif(s) appear to be involved. Recent progress with crystallization of P. furiosus LrpA3 may be very important to confirm molecular details of the archaeal and bacterial Lrp homologues, and as such, LrpA may be a model for further understanding of structure-function relations of this widely distributed class of transcription regulators.

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An Lrp-like Transcriptional Regulator from the Archaeon *Pyrococcus furiosus* Is Negatively Autoregulated

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*J. Biol. Chem.* 2000, 275:38160-38169.
doi: 10.1074/jbc.M005916200 originally published online September 5, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M005916200

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