Mechanism of Autoregulation by an Archaeal Transcriptional Repressor*

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The basal transcription machinery of archaea corresponds to the core components of the eucaryal RNA polymerase II apparatus. Thus, archaea possess a complex multi-subunit RNA polymerase, a TATA box-binding protein and a protein termed transcription factor B (TFB), which is a homologue of eucaryal transcription factor IIB (TFIIB). Intriguingly, archaeal genome sequencing projects have revealed the existence of homologues of bacterial transcriptional regulators. To investigate the mechanism of transcriptional regulation in archaea we have studied one such molecule, Lrs14, a Sulfolobus solfataricus P2 homologue of the bacterial leucine-responsive regulatory protein, Lrp. We find that purified Lrs14 specifically represses the transcription of its own gene in a reconstituted in vitro transcription system. Furthermore, we show that Lrs14 binding sites overlap the basal promoter elements of the Lrs14 promoter and reveal that binding of Lrs14 to these sites prevents promoter recognition by TATA box-binding protein and TFB.

Biochemical dissection of the basal transcription machinery of archaea and the eucaryal RNA polymerase (RNAP) II apparatus has revealed that they are fundamentally related (1–3). In both systems, the initiating step is the recruitment of the TATA box-binding protein (TBP) to the core promoter. In archaea, this is mediated by the direct interaction of TBP with a TATA-box element (4, 5). In eucarya TBP exists within the TFID complex, allowing TBP to be recruited not only by direct TATA-box interactions but also by interactions between TBP-associated factors and other core promoter elements (6, 7). The TBP-DNA complex then recruits archaeal TFB or its eucaryal homologue, TFIIB. In many archaeal promoters TFB interacts with a sequence element, the BRE (TFB-responsive element), situated immediately upstream of the TATA box (8). The cooperative binding of TBP and TFB to the TATA box and BRE has been demonstrated to be of key importance for determining the directional recruitment of the archaeal RNAP to the transcription start site (9). The BRE has also been identified in eucaryal RNAPII promoters as a key determinant of promoter strength (10).

In archaea, the recruitment of the RNAP appears to be via a direct interaction between TFB and the RNAP, and this requires the N-terminal zinc ribbon-containing domain of TFB (11). In eucarya there also appears to be a direct interaction between TFIIB and RNAP II, although it is clear that contacts between TFIIB and the RNAP-associated factor TFIIF are also important for RNAP recruitment. Finally, in eucarya TFIIE and TFIIF play key roles in the late stages of transcriptional initiation and promoter clearance. In contrast, in vitro studies performed on a range of archaeal promoters have demonstrated no requirement for analogous activities in archaea (12). Thus, the archaeal transcription system resembles those factors minimally required for RNAPII transcription. These findings suggest that the last common ancestor of the archaeal and eucaryal lineages possessed a transcription system that used TBP, TFB/TFIIB, and a complex multi-subunit RNAP, a configuration distinct from the simpler bacterial RNAP holoenzyme.

Given the striking similarities between the basal transcription machineries of archaea and eucarya, it might be predicted that similar mechanisms would be employed by these organisms to effect regulated gene expression. However, it is apparent from analysis of archaeal genome sequences that archaea possess a significant number of homologues of bacterial transcriptional regulators (13, 14). How these “bacterial-like” regulators interface with that “eucaryal-like” basal machinery of archaea remains poorly understood. Recently, we characterized MDR1, an archaeal homologue of bacterial metal-dependent transcriptional regulators (15). Experiments performed in vitro and in vivo indicated that MDR1 regulates, in a metal-dependent manner, the expression of a polycistronic transcription unit comprising its own gene and an ABC metal transporter. We showed that MDR1 does not affect promoter recognition by the general transcription factors, TBP and TFB, but effects repression by binding to the promoter downstream of the TATA box and preventing stable recruitment of the RNAP by the TBP/TFB promoter complex (15). A similar mechanism of repression appears to be employed by LrpA, a Pyrococcus furiosus homologue of the bacterial Lrp/AsnC family of transcriptional regulators.2 A recent report described the presence of a Lrp/AsnC family member in Sulfolobus solfataricus P2 (16). This protein, Lrs14, was demonstrated to specifically bind DNA upstream of its own gene. However, as the transcription start site of the gene was not determined, the role, if any, of Lrs14 in transcription remains to be established. In the current work, we demonstrate by using a reconstituted in vitro transcription system that Lrs14 negatively regulates the expression of its own gene. We also characterize the binding sites of Lrs14 and show them

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† The abbreviations used are: RNAP, RNA polymerase; TBP, TATA box-binding protein; PCR, polymerase chain reaction; EMSA, electrophoretic mobility shift assay; PIC, preinitiation complex; BA, bacterial-archaeal; ds, double-stranded; TFB, transcription factor B; TFIIB, transcription factor IIB.

2 A. Brinkman and J. van der Oost, personal communication.
FIG. 1. Purification of Lrs14, identification of its promoter, and establishment of Lrs14-dependent transcriptional repression in vitro. A, Coomassie blue-stained gel of 3 μg of purified 6-His-tagged Lrs14. B, Coomassie blue-stained gels of fractions obtained from chromatography of Lrs14 on a Superox 12 column. The position of elution of molecular weight standards are indicated. C, primer extension analysis of 5 μg of S. solfataricus P2 RNA (left panel) or products of in vitro transcription of pLRPRO (right panel) with oligonucleotide LRSINT. Lanes labeled C, T, A, and G contain dideoxy sequencing reactions primed with LRSINT, and lane P contains the product of primer extension (indicated by a black arrow). D, summary of the primer extension data. The mapped start site is shown with a black arrow, the start codon is underlined, and the putative TATA-element and BRE are boxed. Nucleotide positions relative to the start site are indicated. E, products of in vitro transcription assays performed on the Lrs14 (top panel) and T6 (lower panel) promoters. Reactions contained 0, 125, 250, 500, or 1000 ng of Lrs14.

MATERIALS AND METHODS

Cloning and Expression of Lrs14—The Lrs14 gene was amplified by PCR using Pwo polymerase (Roche Molecular Biochemicals) and oligonucleotide primers LRS5 (5' ATATCCGTTAATCTT 3') and LRS3 (5' GGAAAATTCGAGCTTTCTATCATTTG 3'), and the PCR product was cloned into pTOPO-XL (Invitrogen) to create pLRPRO. The PCR product was also digested with NdeI and XhoI, and the resultant fragment was ligated to NdeI/XhoI-digested pET30a, creating pET-Lrs. The Escherichia coli strain BLR RIL was transformed with pET-Lrs for expression of recombinant hexahistidine-tagged Lrs14. Cells were grown to an OD600 of 0.4, and expression was induced with the addition of isopropyl-1-thio-β-D-galactopyranoside to 1 mM with growth continued for another 3 h. Cells were harvested by centrifugation, resuspended in N300 (50 mM Tris, pH 8.0, 10% glycerol, 300 mM NaCl, 10 mM β-mercaptoethanol), and lysed by sonication. Following clarification by centrifugation, the extract was heated to 75 °C for 30 min and centrifuged to remove denatured protein. Imidazole was added to 15 mM, and the supernatant was applied to a Ni-NTA agarose column (QIAGEN). The column was washed with 10 volumes of N300 plus 40 mM imidazole and 1-mL fractions were collected. These were assayed for the presence of Lrs14 by SDS polyacrylamide gel electrophoresis. Positive fractions were pooled and dialyzed against 200 volumes of N300 overnight. Gel filtration was performed using a Superose 12, 3/20 column attached to a SMART system (Amersham Pharmacia Biotech) at a flow rate of 40 μl/min, with 40-μl fractions collected. Calibration standards were chromatographed according to the manufacturer's instructions.

In Vitro Transcription Reactions—These assays were performed as described previously (11) using either pLRPRO or pT6 (see Ref. 19) as template. Products of in vitro transcription assays were detected by primer extension assays using radiolabeled oligonucleotide primers (T7 primer for pT6 and LRSINT 5' GAAATTTATTACGGTCTAC 3' for pLRPRO).

Primer Extension Analysis of S. solfataricus P2 RNA—A starter culture of S. solfataricus P2 was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (strain 1617) and grown in medium 182 at 75 °C with shaking. Cells were grown to an OD600 of 0.8 and harvested by centrifugation. RNA was prepared using the RNeasy kit (QIAGEN). 5 μg of RNA was annealed to 1 ng of 32P-radio labeled LRSINT, and primer extension was performed as described (20). The products of reverse transcription were electrophoresed on an 8% denaturing polyacrylamide gel alongside a sequence ladder generated using the same oligonucleotide primer and the fmol® system (Promega).

Electrophoretic Mobility Shift Assay (EMSA) and Footprint Analysis—The probes for footprinting were prepared by PCR using T7 and 32P 5'-radiolabeled LRSINT oligonucleotides for studies of the transcribed strand and LRSINT and 32P 5'-radiolabeled T7 for the nontranscribed strand. The oligonucleotides were labeled using T4 polynucleotide kinase and γ-32P-ATP (6000 Ci/mmol; NEN Life Science Products) as described (20). The same radiolabeled primers were used to generate a dideoxynucleotide sequence ladder using the fmol® kit (Promega). DNaseI footprinting was performed as described (11).
EMSAs were performed using annealed oligonucleotide probes as indicated in the figures under conditions described previously (15).

RESULTS

Purified Lrs14 Is a Homodimer in Solution—The open reading frame of \textit{S. solfataricus} Lrs14 was cloned, and the 14-kDa gene product was expressed in \textit{E. coli} as a C-terminally hexahistidine-tagged protein (Fig. 1A). The purified protein was chromatographed over a Superose 12 gel filtration column. By reference to the elution profile of size standards, the protein was determined to have a molecular mass of approximately 30 kDa (Fig. 1B). This suggests that Lrs14 exists principally as a homodimer in solution. This conclusion was supported by cross-linking studies using the homobifunctional cross-linker di-methylsuberimidate (data not shown).

Lrs14 Specifically Represses Its Own Transcription in a Reconstituted in Vitro System—Previous work has demonstrated that Lrs14 binds to regions upstream of its own open reading frame (16). However, the transcription start site and, therefore, the promoter of this gene, have not been experimentally defined. To address this issue, RNA was isolated from \textit{S. solfataricus} Lrs14 in late logarithmic growth phase (\(A_{600} = 0.8\)) and subjected to primer extension analysis. The product of primer extension was electrophoresed adjacent to a sequencing ladder derived using the same radiolabeled primer. The result shown in the left panel of Fig. 1C indicates that transcription starts at the A of the ATG start codon of Lrs14. The putative promoter region of Lrs14 was then amplified by PCR and cloned into pCR-TOPO (Invitrogen) to generate pLRPRO. This plasmid was used as template in a reconstituted archaeal transcription system containing purified \textit{S. acidocaldarius} RNAP and purified recombinant \textit{S. acidocaldarius} TBP and TFB (11). RNA was recovered from the reaction and subjected to primer extension analysis as above (Fig. 1C, right panel; summarized in Fig. 1D). In agreement with the start site detected using RNA isolated from cells, the principal start detected in this assay (shown with a black arrow in Fig. 1C, left panel and Fig. 1D) is found at the A of the start codon for Lrs14, underlined in Fig. 1D. Consensus archaeal TATA-box and BRE elements, the recognition sites for TBP and TFB, respectively, are found 24–37 nucleotides upstream of the mapped start site (Fig. 1D).

Further in vitro transcription assays with the Lrs14 promoter were performed in which varying amounts of recombinant Lrs14 were added. These reactions were assembled on ice prior to incubation at 70 °C to permit transcription. As can be seen in the upper panel of Fig. 1E, Lrs14 represses transcription from its own promoter in a concentration-dependent manner. Importantly, no Lrs14-dependent inhibition of transcription was observed from a control template, that of the T6 promoter of \textit{Sulfolobus shibatae} virus SSV1 (Fig. 1E, lower panel). These data indicate that Lrs14 can specifically repress transcription from its own promoter.

Lrs14 Binding Sites Overlap the TATA Box and BRE—To investigate the mechanism whereby Lrs14 mediates transcriptional repression, we sought to determine the DNA sequences

\textbf{Fig. 2.} \textit{Lrs14 binds its own promoter, overlapping the TATA box and BRE.} A, EMSA with increasing amounts of Lrs14 on a double-stranded oligonucleotide probe comprising nucleotides –4 to –60. B, DNaseI footprinting of 1000 and 100 ng of Lrs14 (lanes 3 and 2) and 20 ng of TBP plus 25 ng of TFB (lane 5) on the coding (left panel) and non-coding strands (right panel) of the Lrs14 promoter. The positions of cleavage points relative to the start site of transcription are indicated, and the brackets indicate the positions of the footprints. C, summary of the DNaseI footprints showing the sequence of the Lrs14 promoter. The start site of transcription is shown with an arrow, and the positions of the presumptive TATA box and BRE are boxed. The black bars indicate the position of the Lrs14-induced DNaseI footprint, and the white rectangles indicate the TBP/TFB-induced footprint. Positions of base pairs relative to the transcription start site are indicated.

EMSAs were performed using annealed oligonucleotide probes as indicated in the figures under conditions described previously (15).
recognized by Lrs14. In agreement with previous reports we found that purified Lrs14 recognized its own promoter in electrophoretic mobility shift assays (16). At least three distinct Lrs14-DNA complexes are detected (Fig. 2A). Competition experiments indicated that these complexes arise from sequence-specific DNA-protein interactions (data not shown and below). DNaseI footprinting analyses were next performed to map the binding sites of Lrs14 on the Lrs14 promoter (Fig. 2B). Notably, Lrs14 binding gave rise to extensive DNaseI footprints extending from −60 to near the start site of transcription. TBP and TFB generated a footprint encompassing the region from −15 to −40, in agreement with the positions of the TATA box and BRE predicted from the primer extension assays (Fig. 1D).

Thus, it is apparent that the binding sites of these two complexes overlap, with the TBP-TFB binding region lying entirely within the region protected by Lrs14 from DNaseI-mediated cleavage (Fig. 2C). These data therefore suggested that Lrs14 might interfere with TATA-box and BRE recognition by TBP and TFB.

**Lrs14 Binds to Multiple Sites in Its Own Promoter**—To further characterize the Lrs14 binding sites a series of deleted derivatives of the Lrs14 promoter were generated and used in the EMSA with the Lrs14 protein. The results shown in Fig. 3A suggest that Lrs14 has at least two distinct binding sites within the region of the promoter protected from cleavage by DNaseI. These sites lie on either side of the TATA box and BRE. To analyze further the binding site lying between −34 and −5, linker-scanning substitutions were introduced, and the ability of these altered DNA molecules to be recognized by Lrs14 was assessed by EMSA. As seen in Fig. 3B, substitution of G residues for the natural sequences from −30 to −26 (probe F1) abrogates Lrs14 binding, whereas substitution of residues −25 to −22 (probe F2) reduces Lrs14 binding. In contrast, substitution of residues −18 to −16 (probe F3) has no significant effect on binding. The importance of the sequences from −30 to −26 is particularly noteworthy as they lie within the region of the promoter predicted to function as the TATA box. Thus, it appears that Lrs14 and TBP recognize overlapping sequence elements in the Lrs14 promoter. We tested this hypothesis by determining the ability of the TBP-TFB ternary complex to form on oligonucleotides containing the F1 substitutions. In agreement with the predicted position of the TATA box, we find that substitution of G-rich sequences at positions −30 to −26 abrogate TBP-TFB-DNA complex formation (Fig. 3C).

**Binding of Lrs14 and TBP-TFB to the Lrs14 Promoter Are Mutually Exclusive Events**—The transcription assays shown in Fig. 1E were set up by adding the promoter DNA to a reaction already containing Lrs14, TBP, TFB, and RNAP. Given the overlap detected above between the sequences involved in preinitiation complex (PIC) formation and Lrs14 binding, we decided to test whether Lrs14 was able to disrupt a pre-formed PIC. Accordingly, order of addition transcription experiments were performed in which PIC was formed by addition of TBP, TFB, and RNAP to template DNA for varying time periods prior to challenge with Lrs14 (Fig. 4A). Nucleoside triphosphates were then added to initiate transcription, and yields of transcript were detected by primer extension. The results of this assay (Fig. 4A) indicate that if the PIC is formed prior to addition of Lrs14 then no repression of transcription occurs. In contrast, if Lrs14 is added at the same time as or prior to PIC formation then transcription is strongly repressed. These data therefore suggest that, although Lrs14 is unable to displace the PIC from DNA, the converse is also true; PIC components cannot displace Lrs14 from the promoter. Identical results were obtained when unfractionated extract prepared from *S. solfataricus* P2 was used as a source of basal transcription factors and RNAP (data not shown).

To investigate the competition between Lrs14 and the PIC further, DNaseI footprinting assays were performed to determine whether there was any competition between DNA binding by TBP/TFB and by Lrs14. Binding reactions were performed in which radiolabeled probe was pre-incubated with either TBP/TFB or Lrs14 for varying lengths of time. Following this pre-incubation, the TFB/TBP reactions were supplemented with Lrs14, and the Lrs14 reactions had TBP/TFB added. No-
tably, neither Lrs14 nor TBP/TFB could supplant the opposing factor-DNA complex, indicating that both complexes are stable and resistant to challenge (Fig. 4B). Similar results were obtained with EMSAs performed on reactions in which Lrs14 had been added to reactions containing either naked DNA or pre-formed TBP/TFB-DNA complexes (Fig. 4C). At the various time points indicated in Fig. 4C, reactions were loaded on a running non-denaturing polyacrylamide gel. As the gel was loaded over the 30-min time course of the experiment, the complexes migrated for differing times, resulting in the slanted appearance of the gel. The lower panel of this figure is a schematic interpretation of the upper panel. Complexes containing TBP/TFB are shown as black ovals, Lrs14-containing complexes are shown as open ovals, and unbound DNA is shown as gray ovals.

**FIG. 4.** Complex formation on the Lrs14 promoter is resistant to challenge. A, in vitro transcription assays performed using the reconstituted transcription system on the Lrs14 promoter. The various components were added at the indicated times into a reaction that had been pre-warmed to 70 °C. NTPs were added 10 min later, and the transcription reaction was continued for another 10 min. RNA was recovered and subjected to primer extension analysis as described under “Materials and Methods,” prior to electrophoresis on 8% denaturing polyacrylamide gel. B, DNaseI footprinting analysis of TBP/TFB and Lrs14 binding to the transcribed strand of the Lrs14 promoter. The indicated proteins (20 ng of TBP and 25 ng of TFB or 500 ng of Lrs14) were added at the indicated times into a 15-min binding reaction at 48 °C. At 15 min DNaseI was added, and samples were processed as described previously (11). C, upper panel, EMSA performed in which either no protein or 20 ng of TBP and 25 ng of TFB were incubated with radiolabeled ds oligonucleotide probe A (see Fig. 3A) for 10 min. 500 ng of Lrs14 were then added, and the incubation was continued for the indicated times prior to loading on a running 6% non-denaturing polyacrylamide gel. As the gel was loaded over the 30-min time course of the experiment, the complexes migrated for differing times, resulting in the slanted appearance of the gel. The lower panel of this figure is a schematic interpretation of the upper panel. Complexes containing TBP/TFB are shown as black ovals, Lrs14-containing complexes are shown as open ovals, and unbound DNA is shown as gray ovals.

**DISCUSSION**

Previous work has identified Lrs14 as a protein that binds upstream of its own open reading frame (16). We show that the Lrs14 binding sites actually overlap the basal promoter elements important for mediating Lrs14 gene transcription. Moreover, we present evidence that Lrs14 autoregulates its own expression by controlling the accessibility of the TATA box and BRE of the Lrs14 gene to the basal transcription factors, TBP and TFB. This mode of transcriptional repression by Lrs14 is highly distinct from that mediated by the archaeal metal-dependent repressor, MDR1, which binds to consecutive operators downstream of the TATA box, allowing TBP and TFB to bind to the core promoter elements but blocking the subsequent recruitment of RNAP by the TBP-TFB promoter ternary complex (15).

It is tempting to speculate that the different mechanisms of repression by MDR1 and Lrs14 reflect the differing biological roles of the genes that they regulate. In the case of MDR1 it regulates expression of a polycistronic transcription unit containing its own gene and a metal-importing ABC transporter system. By having TBP and TFB pre-bound to the promoter, the system is poised to rapidly recruit RNAP and initiate transcription in response to decreases in intracellular metal ion concentration. This ability to respond rapidly may be of crucial importance when the cell needs to maintain suitable levels of vital metal ion co-factors despite constantly changing environmental conditions. In the case of Lrs14, the protein is autoregulating expression of a monocistronic transcription unit contain-
ing only its own gene. Although the biology of Lrs14 is currently less clear, our data suggest that the cell does not need to mount a rapid response to regulate levels of the Lrs14 protein. It is tempting to speculate that downstream targets of Lrs14 may be regulated by a mechanism akin to MDR1 regulation.

Our current work adds to the evidence that regulation of archaeal gene expression is mediated by molecules that are generally more closely related to bacterial than to eucaryal transcriptional repressors. Indeed, a recent analysis indicates that the abundance of genes encoding predicted bacterial-like transcriptional regulators in archaeal genomes is roughly equivalent to those in bacterial genomes (13). For example, 3.5 and 2% of the predicted open reading frames of the archaeon *Archaeoglobus fulgidus* and the bacterium *Aquifex aeolicus*, respectively, contain bacterial-like helix turn helix motifs (13). As this class of regulators is found in both archaeal and bacterial domains, we shall refer to these molecules as bacterial-archaeal (BA) regulators. The presence of BA regulators in both domains could in part reflect lateral gene transfer events having taken place between bacteria and archaea. However, the extent of the similarity between the BA regulators in the two domains is more consistent with a second interpretation, that the BA regulators were established prior to the divergence of these two lineages. This possibility would account for the existence of archaeal-specific families of helix turn helix-containing factors that are nonetheless more closely related to bacterial than eucaryal helix turn helix-containing factors (13).

It is widely accepted that the divergence of eucaryal and archaeal lineages occurred following the divergence of the bacteria (17). An important inference from the above proposal is therefore that the last common ancestor of archaea and eucarya possessed a transcription machinery with a basal apparatus akin to that found in present day archaea combined with BA-type regulators. If this is correct, then it raises the question of why there is a paucity of BA-type regulators in eucarya. An attractive explanation is that as the eucarya evolved ever-increasing genome size and the consequential requirement for higher order DNA compaction systems, the generally repressive role of chromatin became more and more dominant. There may then have been considerable pressure for eucarya to develop novel chromatin-modulatory systems. This may have led to the loss of the simple BA regulators and their replacement by the present eucaryal system in which transcriptional regulation and chromatin modification are inextricably interwoven (18). Clearly, further investigations into the biological roles and mechanisms of actions of archaeal, bacterial, and eucaryal transcriptional regulators will shed light on the evolution of gene regulatory processes.

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