Control of Ribosomal Protein L1 Synthesis in Mesophilic and Thermophilic Archaea

Alexander Kraft, Christina Lutz, Arno Lingenhel, Peter Gröbner and Wolfgang Piendl

Institute of Medical Chemistry and Biochemistry, University of Innsbruck, A-6020 Innsbruck, Austria

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ABSTRACT

The mechanisms for the control of ribosomal protein synthesis have been characterized in detail in Eukarya and in Bacteria. In Archaea, only the regulation of the MvaL1 operon (encoding ribosomal proteins MvaL1, MvaL10, and MvaL12) of the mesophilic Methanococcus vannielii has been extensively investigated. As in Bacteria, regulation takes place at the level of translation. The regulator protein MvaL1 binds preferentially to its binding site on the 23S rRNA, and, when in excess, binds to the regulatory target site on its mRNA and thus inhibits translation of all three cistrons of the operon. The regulatory binding site on the mRNA, a structural mimic of the respective binding site on the 23S rRNA, is located within the structural gene about 30 nucleotides downstream of the ATG start codon. MvaL1 blocks a step before or at the formation of the first peptide bond of MvaL1. Here we demonstrate that a similar regulatory mechanism exists in the thermophilic M. thermolithotrophicus and M. jannaschii. The L1 gene is cotranscribed together with the L10 and L11 gene, in all genera of the Euryarchaeota branch of the Archaea studied so far. A potential regulatory L1 binding site located within the structural gene, as in Methanococcus, was found in M. methylotrophus thermautotrophicum and in Pyrococcus horikoshii. In contrast, in Archaeoglobus fulgidus a typical L1 binding site is located in the untranslated leader of the L1 gene as described for the halophilic Archaea. In Sulfolobus, a member of the Crenarchaeota, the L1 gene is part of a long transcript (encoding SecE, NusG, L11, L1, L10, L12). A previously suggested regulatory L1 target site located within the L11 structural gene could not be confirmed as an L1 binding site.

The translational apparatus of the Archaea appears to be a mosaic of bacterial and eukaryal features (Amils et al. 1993; Dennis 1997; Bell and Jackson 1998). The complete sequence of the Methanococcus jannaschii genome provided the first clear and comprehensive picture of archaeal organization of the genes for the components of the translational apparatus (Bult et al. 1996). A total of 60 genes for ribosomal proteins could be identified, a value slightly higher than that found in Bacteria. In general, the primary structures of archaeal ribosomal proteins are more related to their eukaryal than to their bacterial counterparts. However, the genes for the ribosomal proteins are arranged in operons as in Bacteria; even the order of the genes is similar to that found in Bacteria (Ramirez et al. 1993). The regulation of expression of ribosomal proteins has been investigated extensively in Escherichia coli (Zengel and Lindahl 1994). Most of the ribosomal protein operons contain a cistron encoding a ribosomal protein that can bind either to a specific site on the rRNA or to a site on its own mRNA, thereby inhibiting translation of the operon. The coordinated expression of all genes present in a polycistronic mRNA is guaranteed by translational coupling, i.e., translation of an upstream cistron is required to initiate the translation of the distal cistrons (Nomura et al. 1984). In Archaea, only the regulation of the MvaL1 operon (encoding ribosomal proteins MvaL1, MvaL10, and MvaL12) of the mesophilic M. vannielii has been studied in detail (Hanner et al. 1994; Mayer et al. 1998). The expression of the proteins from this operon is subject to a bacteria-like autogenous control. MvaL1, the homolog of the regulatory protein L1 encoded by the bicistronic L11-L1 operon of E. coli, was identified as the autoregulator of the MvaL1 operon. In contrast to all other translational regulatory systems studied so far, the binding site of the translational repressor MvaL1, a structural mimic of the 23S rRNA binding site (Figure 1), is located inside the MvaL1 coding region, about 30 bases downstream of the initiation codon. Binding of MvaL1 to its own mRNA has been demonstrated. Furthermore, we could show that MvaL1 inhibits translation at or a step before the formation of the first peptide bond. The translation of the distal MvaL10 and MvaL12 cistrons is coupled to that of the MvaL1 cistron.

Apart from mesophilic species such as M. vannielii, the genus Methanococcus comprises moderately thermophilic species such as M. thermolithotrophicus (optimal growth temperature 65°C; Huber et al. 1982) and hyperthermophilic species such as M. jannaschii (optimal growth temperature 85°C; Jones et al. 1983). In the MethL1...
sequence (GenBank accession no. AF044919) and in the M. jannaschii gene (Bult et al. 1996) potential L1 binding sites were discovered suggesting a similar mechanism of translational regulation in mesophilic and thermophilic Methanococci (Köhrer et al. 1998). Here we provide experimental evidence for the translational control of ribosomal L1 synthesis in M. thermolithotrophicus and M. jannaschii. Furthermore, we suggest a similar translational regulatory mechanism for the corresponding L1 genes from the Eurarchaeota Methanothermobacterthermoautotrophicum (Smit et al. 1997) and Pyrococcus horikoshii (Kawarabayasi et al. 1998), as typical regulatory L1 binding sites are located inside their L1 genes. In contrast, analysis of the L1 sequence of Archaeoglobus fulgidus (Klenk et al. 1997) revealed a potential regulatory L1 binding site located in the untranslated leader of the L1 gene, as described for halophilic Archaea (Shimmin and Dennis 1994). In Sulfolobus acidocaldarius and S. solfataricus, members of the Crenarchaeota branch of the Archaea, the L1 genes appear to be part of long transcripts (encoding SecE, NusG, L1, L11, L10, L12). A previously suggested regulatory L1 binding site located inside the L11 gene of S. acidocaldarius (Ramirez et al. 1994) could not be confirmed by filter-binding studies.

Materials and Methods

Strains and growth conditions: The E. coli strain TG2 (Δ(lacproA) supF thi r era (F’trd36 proA 8 lacZΔM15)) (Gibson 1984) was used for the propagation of plasmids. E. coli BL21(DE3) (F- hsdS gal ompT (Δd57 ind1 Sm7 nin5 lacUV5-T7 gene 1)) (Studier and Moffat 1986) was the host strain for the production of recombinant ribosomal proteins. Strains were grown in Luria-Bertani (LB) medium. Ampicillin and kanamycin were added to 100 and 50 μg/ml, respectively, when required for the maintenance of plasmids. Plasmids used in this study are listed in Table 1.

Plasmid constructions: Genomic DNA from M. thermolithotrophicus was prepared as described previously (Jarsh et al. 1983). DNA fragments were eluted from agarose gels using the QIAquick Gel Extraction Kit (QIAGEN Inc., Valencia, CA) and plasmids were purified using the QIaprep Spin Miniprep Kit (QIAGEN Inc.), following manufacturer’s instructions. For the PCR reactions, the Advantage cDNA Polymerase Mix (CLONTECH, Palo Alto, CA), which provides an antibody-mediated “hot start” and a proofreading activity, was used. The PCR protocol and the cloning of the PCR products via pUC18 are detailed in Hanner et al. (1994). The correct sequences of the cloned PCR products were confirmed by double-stranded sequencing.

To clone the SacL1 gene from S. acidocaldarius into the high-level expression vector pET11a, a 0.7-kb DNA fragment containing an NdeI site (including the ATG start codon) and a BamHI site was created by PCR using genomic DNA from S. acidocaldarius as template and inserted in the corresponding sites of the vector to give plasmid pSacl1.4. The construction of the pl expression cassettes used as templates in in vitro regulation studies and as templates for in vitro T7 RNA polymerase transcription is summarized in Table 1. The M5 mutations were introduced in the L1 binding sites on the mRNA of M. jannaschii and M. thermolithotrophicus as described for M. vannilli using a PCR-based site-directed mutagenesis method. Owing to the high degree of sequence conservation of the L1 binding sites, the same primers as for the construction of plasmid L5M5 (Mayer et al. 1998) could be used.

Overproduction and purification of L1 proteins: In general, the L1 proteins used in this study were overproduced in E. coli BL21(DE3) transformed with the relevant plasmid as listed in Table 1. In Methanococcus and Sulfolobus, the codons mainly used for arginine are AGG and AGA, which are the less frequently used codons in E. coli. To obtain maximal yields and to avoid a potential misincorporation of amino acids (Caldon et al. 1996), the E. coli host was cotransformd with pUB5520, a plasmid that carries the gene for the rare tRNA
t and tRNA
t (Brinkmann et al. 1989). Overproduction and purification by ion exchange chromatography of MvaL1 (Mayer et al. 1998), MthL1, and MjaL1 (Köhrer et al. 1998) have been reported previously. Purification of SacL1 was performed as described for SsoL1 (Köhrer et al. 1998).

In vitro protein synthesis: In vitro transcription-translation reactions were carried out as described (Köhrer et al. 1996). The S30 crude extract containing T7 RNA polymerase was prepared from E. coli BL21(DE3). Reaction mixtures contained 0.8 μg of plasmid template, 7.5 μl of low-molecular-weight mixture (Pratt 1984), 2 μl of 0.1 M magnesium acetate, 2 μl of S30 extract, 1 μl of rifampicin (10 mg/ml in methanol), and 10 μl of 1μM[methionine (1200 Ci/ml; New England Nuclear Corp., Boston) in a total volume of 30 μl of 10 mm Tris-acetate buffer (pH 7.0). Reaction mixtures were incubated at 37° for 35 min and then chased for 10 min with 10 μl of nonradioactive methionine (8 mg/ml). The radiolabeled proteins were separated on 18% polyacrylamide/SDS gels (Laemmli 1970). After autoradiography, bands were quantified with a scanner (Vilber Lourmat, Marne-la-Vallee, France; Bio-Imaging).

Filter-binding assays: The plasmids used as templates to produce rRNA and mRNA fragments containing the L1-binding sites are listed in Table 1. Uniformly 32P-labeled RNA fragments were synthesized in vitro from templates linearized with HindIII in the presence of [α-32P]UTP (800 Ci/mmole) using the MAXIscript T7 kit (Ambion Inc., Austin, TX). Unincorporated nucleotides were removed with the QIAquick Nucleotide Removal Kit (QIAGEN Inc.). The purity and integrity of the transcripts were confirmed by electrophoresis on 8% polyacrylamide SDS gels containing 8 M urea. The affinity of L1 proteins to RNA-binding sites was determined by a nitrocellulose-filter-binding assay as described in detail by Köhrer et al. (1998). The binding buffer contained 50 mm Tris/ HCl, pH 7.6, 20 mm MgCl2, 500 mm KCl, 1 mm 2-mercaptoethanol, and 0.04% BSA.

RNA structure prediction: RNA secondary-structure prediction was performed using two different computer packages, RNAfold (Mathur and Wenberg 1996) and STAR (Abramowicz et al. 1990). RNAdraw uses a minimum energy structure prediction algorithm ported from the program RNAfold included in the Vienna RNA package (Hofacker et al. 1994), whereas a genetic algorithm for RNA folding simulations is used in the package STAR (van Batemburg et al. 1995).

Nomenclature of ribosomal proteins: Throughout the article we use the designation for ribosomal proteins as proposed by Wittmann-Liebold et al. (1990) to indicate from which organism the ribosomal proteins originate: Atu (A. fulgidus), Mja (M. jannaschii), Mth (M. thermolithotrophicus), Mva (M. vannilli), Sac (S. acidocaldarius), and Sso (S. solfataricus).

Results

Interaction of ribosomal proteins L1 from the thermophilic M. jannaschii and M. thermolithotrophicus with...
TABLE 1
Plasmids used in this study

<table>
<thead>
<tr>
<th>Description/application</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td><strong>Vectors</strong></td>
<td></td>
</tr>
<tr>
<td>pBluescript KS/ SK Ap', lac' IPOZ' α, T7 promoter</td>
<td>Short et al. (1988)</td>
</tr>
<tr>
<td>pUC18/19 Ap', lac' IPOZ' α</td>
<td>Yanisch-Perron et al. (1985)</td>
</tr>
<tr>
<td><strong>Recombinant plasmids</strong></td>
<td></td>
</tr>
<tr>
<td>pET11a constructs</td>
<td></td>
</tr>
<tr>
<td>Overproduction of MjaL1</td>
<td>Köh rer et al. (1998)</td>
</tr>
<tr>
<td>Overproduction of MthL1</td>
<td>Köh rer et al. (1998)</td>
</tr>
<tr>
<td>Overproduction of MvaL1 M5</td>
<td>Mayer et al. (1998)</td>
</tr>
<tr>
<td>Insert: 0.7-kb NdeI-BamHI PCR fragment (template: genomic DNA of S. acidocaldarius), containing the SacL1 gene; overproduction of SacL1</td>
<td>This study</td>
</tr>
<tr>
<td><strong>pBluescript expression constructs</strong></td>
<td></td>
</tr>
<tr>
<td>Insert: 0.8-kb SspI-AflIII fragment containing the MjaL1 gene; in vitro synthesis of MjaL1 (regulation studies)</td>
<td>This study</td>
</tr>
<tr>
<td>Derivative of pMjaL1.3 with M5 mutation</td>
<td>This study</td>
</tr>
<tr>
<td>Derivative of pMvaL1.3 with M5 mutation</td>
<td>This study</td>
</tr>
<tr>
<td><strong>pBluescript transcription constructs</strong></td>
<td></td>
</tr>
<tr>
<td>Insert: 0.15-kb SacI-HindIII PCR fragment (template: genomic DNA of M. thermolithotrophicus) containing the MthL1 binding site on 23S rRNA; in vitro synthesis of a 150-nt-long 23S rRNA fragment</td>
<td>This study</td>
</tr>
<tr>
<td>Insert: 0.16-kb SacI-HindIII PCR fragment (template: genomic DNA of S. acidocaldarius) containing the SacL1 binding site on 23S rRNA; in vitro synthesis of a 160-nt-long 23S rRNA fragment</td>
<td>This study</td>
</tr>
<tr>
<td>Insert: 0.25-kb SacI-HindIII PCR fragment (template: genomic DNA of M. thermolithotrophicus) containing the MthL1 binding site on mRNA; in vitro synthesis of a 250-nt-long mRNA fragment</td>
<td>This study</td>
</tr>
<tr>
<td>Derivative of pMthL1.Tk with M5 mutation</td>
<td>This study</td>
</tr>
<tr>
<td>Derivative of pMvaL1.10 with M5 mutation</td>
<td>This study</td>
</tr>
<tr>
<td>Insert: 0.25-kb SacI-HindIII PCR fragment (template: genomic DNA of S. acidocaldarius) containing the potential SacL1 binding site on L11 mRNA; in vitro synthesis of a 250-nt-long mRNA fragment</td>
<td>This study</td>
</tr>
<tr>
<td><strong>pMva16S-S8BS.3</strong> in vitro synthesis of a 220-nt-long M. vannii mRNA fragment containing the MvaS8 binding site</td>
<td>Köh rer et al. (1998)</td>
</tr>
</tbody>
</table>

**their own mRNA:** In a previous study we had shown that ribosomal proteins MjaL1 and MthL1 bind the regulatory MvaL1-binding site on the mRNA of M. vannii with rather high affinity (Köh rer et al. 1998). Analysis of the mRNAs of MjaL1 and MthL1 revealed structures very similar to the regulatory MvaL1-binding site on the mRNA of M. vannii (Figure 1A). The affinity of MjaL1 and MthL1 for their own mRNA was compared to that for their own 23S rRNA. To minimize potential non-specific interactions, a binding buffer containing 500 mm KCl was chosen (Köh rer et al. 1998). A 250-nucleotide transcript encompassing the MthL1 mRNA-binding site
Figure 1.—Comparison of (A) mRNA and (B) 23S rRNA
binding sites for ribosomal protein L1 of different Methano-
coccus species. The structures of M. thermolithotrophicus are
shown. Nucleotides different in M. jannaschii are boxed, nucle-
etides different in M. vannielii are circled. Arrows indicate nucleotides altered by site-specific mutagenesis in mutants M5. Base numberings in A are taken from the translation start. Helices in B are numbered according to the E. coli nomenclature. Highly conserved nucleotides are shaded.

Figure 2.—Binding curves for the interaction of ribosomal proteins MthL1 (solid lines, solid symbols) and, for comparison, MvaL1 (broken lines, open symbols) with their homolo-
gous 23S rRNA (▲, △), wild-type mRNA (●, ○), and mutated M5 mRNA (■, □) binding sites. A 16S rRNA fragment (solid line, X) was used as a control. Representative experiments are shown. Values of RNA retained on the filter in the absence of protein (2–5% for the rRNA fragments and 7.5–10% for the mRNA fragments) were subtracted before the data were blotted.

Ribosomal L1 proteins from thermophilic Methano-
coccus species inhibit their own synthesis in vitro: A coupled transcription-translation system based on E. coli S30 crude extract was set up to investigate the regulatory function of the ribosomal ribosomal proteins. Plas-
mids pMvaL1.35 and pMvaL1.35M5 were used as a template to compare the inhibitory effects of different L1 proteins. To examine its functional role as a transla-
tional regulator, purified L1 from thermophilic and mesophilic organisms was added to the system. The addition of MjaL1 resulted in a much stronger inhibition of L1 synthesis than the addition of mesophilic MvaL1 (Figure 3). Forty picomoles of exogenously

| Table 2
<p>| Binding of ribosomal protein L1 from the thermophilic and mesophilic Methanococcus species to their homologous 23S rRNA and mRNA |
|---------------------------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Protein</th>
<th>RNA</th>
<th>$K_d$ (M)</th>
<th>Saturation level (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MjaL1</td>
<td>23S rRNA</td>
<td>$1.2 \times 10^{-10}$</td>
<td>60–90</td>
</tr>
<tr>
<td></td>
<td>mRNA-WT</td>
<td>$3.9 \times 10^{-9}$</td>
<td>36–40</td>
</tr>
<tr>
<td></td>
<td>mRNA-M5 mutant</td>
<td>$2.5 \times 10^{-7}$</td>
<td>67</td>
</tr>
<tr>
<td>MthL1</td>
<td>23S rRNA</td>
<td>$3.1 \times 10^{-10}$</td>
<td>60–65</td>
</tr>
<tr>
<td></td>
<td>mRNA-WT</td>
<td>$7.2 \times 10^{-9}$</td>
<td>60–70</td>
</tr>
<tr>
<td></td>
<td>mRNA-M5 mutant</td>
<td>$3 \times 10^{-8}$</td>
<td>80–90</td>
</tr>
<tr>
<td>MvaL1</td>
<td>23S rRNA</td>
<td>$5 \times 10^{-9}$</td>
<td>39–65</td>
</tr>
<tr>
<td></td>
<td>mRNA-WT</td>
<td>$1.5 \times 10^{-7}$</td>
<td>52–58</td>
</tr>
<tr>
<td></td>
<td>mRNA-M5 mutant</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

(synthesized from template pMthL1.Tk), the corre-
sponding fragment encompassing the M5 mutation thereof, and a 150-nucleotide fragment containing the 23S rRNA binding site from M. thermolithotrophicus (syn-
thesized from template pMthL1.23S) were used to study the MthL1-RNA interactions. The 220-nucleotide 16S rRNA fragment (synthesized from plasmid pMva16S-
S8BS.3) served as a control to identify nonspecific RNA-protein interactions. For comparison, binding of MvaL1 to its mRNA and rRNA was determined in the same set of experiments. The results of these binding studies are shown in Figure 2. Similarly, the affinity of MjaL1 to its own mRNA and rRNA was determined (data not shown). The average dissociation constants for the inter-
action of MjaL1, MthL1, and MvaL1 with their homolo-
gous rRNA and mRNA fragments are summarized in Table 2. The apparent dissociation constants of the ho-
logous MjaL1-rRNA complex ($K_d: 1.2 \times 10^{-10}$ M) and the MthL1-rRNA complex ($K_d: 3.1 \times 10^{-10}$ M) are virtually identical and in good agreement with the disso-
ciation constants determined for the complexes of the same proteins with heterologous 23S rRNA fragments derived from M. vannielii (Köhler et al. 1998). The affinities of both MjaL1 and MthL1 to their specific binding site on their mRNA are more than 20-fold lower. As shown before (Mayer et al. 1998) and confirmed in the control experiments shown here (Figure 2), MvaL1 binds to its specific RNA target sites with a much lower affinity than L1 proteins derived from thermophilic spe-
cies. In contrast to MvaL1, MjaL1 and MthL1 do not bind to their specific mRNA target site carrying the nonregu-
latory M5 mutant in a highly specific manner, as com-
pared to the noncognate 16S rRNA fragment (Figure 2; Table 2).
added MjaL1 reduced the protein synthesis to ~50% (MvaL1: 70%); 80 pmol of MjaL1 decreased the L1 synthesis to ~30% of the initial value (MvaL1: 50%). The same effect was observed when L1 of the thermophilic M. thermolithotrophicus was added to the system (data not shown). When pMvaL1.35M5 (carrying the nonregulatory M5 mutation; Mayer et al. 1998) was used as a template, neither the addition of a thermophilic L1 protein nor the addition of a mesophilic L1 protein affected the in vitro protein synthesis significantly (Figure 3B). A slight decrease of protein synthesis at high L1 concentrations (120 pmol and higher) was considered to be an artifact of the in vitro system rather than a specific regulation of the M5 mutant (Figure 3A, lane 4). Identical results were obtained when the experiments were repeated using homologous DNA as a template (data not shown).

**Putative regulatory L1 binding sites on the L1-mRNA in Euryarchaeota:** Next, we wanted to investigate whether the autogenous translational regulation of the L1 operon, which is characterized by the localization of the regulatory L1 target site within the structural L1 gene, is a characteristic of Methanococcus or whether it is a regulatory system widespread among the Archaea. The experimentally confirmed regulatory L1 binding sites in Methanococcus and E. coli (Kearney and Nomura 1987), both structural mimics of the respective binding site on 23S rRNA, contain the nucleotides essential for L1 binding (shaded in Figure 1), but differ from the 23S rRNA site in that the central asymmetric loop is 5–6 nucleotides (nt) smaller than the corresponding loop in 23S rRNA (Figure 1).

Using computer programs RNAdraw and STAR, RNA secondary structure analyses were performed on the L1 mRNA of A. fulgidus and on the L11 and L1 mRNA of M. b. thermautotrophicum and P. horikoshii and their respective untranslated leader sequences. A structure that resembles a regulatory L1 target site was found in each of the three species. The potential L1 binding sites of P. horikoshii and of M. b. thermautotrophicum are located within the structural L1 gene, 34 and 25 nucleotides, respectively, downstream of the initiation codon (Figure 4, A and B). The localization is very similar to that in Methanococcus, where the 6-bp helix, which we consider an essential element of the L1 binding site, commences 28 nt downstream of the start codon (Figure 1A). In contrast, the potential L1 regulatory target site of A. fulgidus is located in the untranslated leader sequence of the Aful1 gene. As shown in Figure 4, C and D, the 6-bp helix commences 10 nucleotides upstream of the start codon; its location is thus identical to the suggested L1 regulatory site of Halobacterium cutirubrum (Shimmin and Dennis 1989). No other secondary structures that fulfill the criteria of an L1 binding site could be detected in the mRNAs examined.

**In Sulfolobus, a previously suggested regulatory L1 binding site does not bind L1:** On the basis of the high degree of similarity to the L1 binding site on the 23S rRNA, a putative regulatory L1 binding site was suggested previously in the Crenarchaeon S. acidocaldarius (Ramirez et al. 1994) and a similar motif could be identified in S. solfataricus (Figure 5). These potential L1 binding sites are located within the L11 gene 27 bases downstream of the start codon, the same distance as between the L1 start codon and the experimentally confirmed regulatory L1 target site of the Methanococcus species described in this work. Therefore, we decided to test in vitro the binding capacity of both SacL1 and SsoL1 to their putative binding sites in the L11 mRNA as well as to the respective binding sites in 23S rRNA. To study the SacL1-mRNA interaction, we used a 250-nt-long transcript encompassing the putative L1 binding site, which was transcribed from plasmid pSacL1.1.Tk. pSacL1.23S was used to synthesize the 23S rRNA binding site of SacL1. A 16S rRNA fragment (template pMva16S-S8BS.3) was used as a control for unspecific binding. Figure 6 shows the result of a typical filter-binding assay of SacL1 to the rRNA and mRNA transcripts. The appar-
ent dissociation constant of the SacL1/23S rRNA complex is $5.3 \times 10^{-11}$ M. No significant binding of SacL1 to the SacL11-mRNA transcript could be detected. Similar experiments were performed with SsoL1 and the respective SsoL11-mRNA and 23S rRNA fragments. The result was the same: SsoL1 binds the 23S rRNA fragment with high affinity ($K_d:1 \times 10^{-10}$ M), but does not bind the SsoL11-mRNA fragment.

**DISCUSSION**

**Identification of the authentic initiation codons of archaeal L1 proteins:** When nucleotide sequences encoding ribosomal proteins are analyzed for potential regulatory motifs and structures, the identification of the authentic initiation codon of the relevant gene is essential. In the case of A. fulgidus, M. b. thermoautotrophicum, M. jannaschii, and P. horikoshii, where the complete genome sequences are available, the first in-frame AUG, GUG, or UUG codon seems to have been identified, in general, as a putative initiation codon of the open reading frame. In methanogenic Archaea, as in Bacteria, initiation codons are preceded at the appropriate distance of 3-10 nucleotides by at least a four-base-long ribosome binding sequence complementary to the pyrimidine-rich sequence at the 3' end of 16S rRNA. Dennis (1997) suggested that many of the assignments of putative start codons that lack a Shine-Dalgarno sequence may be incorrect. As for archaeal ribosomal L1 proteins, an incorrect assignation of the initiation codon has been shown for L1 from M. jannaschii. The assigned AUG initiation codon is not preceded by any Shine-Dalgarno-like sequence, whereas the second in-frame AUG triplet is (Tishchenko et al. 1998). Similarly, we have reexamined the translation initiation region of the L1 genes from A. fulgidus, M. b. thermoautotrophicum, and P. horikoshii. As illustrated in Figure 4, A and B, we suggest the second in-frame AUG triplet as the start codon of the L1 genes of M. b. thermoautotrophicum, and P. horikoshii, as both are preceded by good Shine-Dalgarno sequences, whereas no ribosomal loading site could be detected upstream of the first in-frame AUG codon. Our notion is supported by the alignment of the N termini of the archaeal L1 proteins (Figure 7). The short versions of the L1 proteins we suggest show high similarity to other archaeal L1 proteins. For the L1 gene of S. solfataricus, a Shine-Dalgarno sequence overlapping with the start codon has been reported (Ramirez et al. 1994). It is difficult to
Imagine that this region of the mRNA interacts simultaneously with both the 3’ end of the 16S rRNA and the initiator tRNA. Again, the second in-frame GUG codon could be the authentic start codon.

**Transcriptional organization of the archael rRNA genes:** The order of the genes for ribosomal proteins is highly conserved in Bacteria and Archaea (Wächt er shäuser 1998). A good example for this is the cluster encoding, in the following order, ribosomal proteins L11, L1, L10, and L12. In A. fulgidus (Klenk et al. 1997) and Methanococcus (Baier et al. 1990; Bult et al. 1996), unlike all prokaryotes studied so far, the L11 gene is not located upstream of the L1 gene. Transcription analyses in M. vannii (Baier et al. 1990) and halophilic Archaea (Shimmin and Dennis 1996) revealed a tricistronic L1-L10-L12 mRNA. No transcription analyses have been reported yet for ribosomal protein L1 operons of other Euryarchaeota. But we assume that in M. b. thermotrophicum and P. horikoshii, as in Methanococcus and halophilic Archaea, the L1 gene is cotranscribed together with the L10 and L12 genes and that the preceding L11 gene represents (or is part of) a separate transcription unit. In P. horikoshii, a motif AAAAAAGTTTAAAA is located 60 bp upstream of the initiation codon, which fulfills the criteria of a good transcription initiation site (Suckow et al. 1998). In M. b. thermotrophicum, a TATA box-like element (TTTATA), which could be a promoter element, is located 67 bp upstream of the initiation codon. The L1-L10-L12 tricistronic transcript might be a characteristic of the Euryarchaeota.

In the Crenarchaeota branch of the Archaea, the L1 gene organization was studied only in two Sulfolobus species. In S. solfataricus, secE, nusG, and the L11, L1, L10, and L12 genes are clustered. From the short distance of 1-5 nucleotides between the start/stop codons it was concluded that these genes are cotranscribed (Geiger et al. 1997). An identical gene cluster was identified in S. acidocaldarius. Northern blotting and S1 mapping experiments revealed a L11-L10-L12 tetrastronic transcript (Ramir ez et al. 1994). Thus the promoter for this tetrameronic transcript is located within nusG. The suggested promoter box A is 31 and 38 bp, respectively, upstream of the mapped transcription start-sites. In contrast, previous analyses of the Sulfolobus promoter revealed that efficient transcription was dependent on a distance of about 26 nucleotides between TATA box and transcription start-site (Reiter et al. 1990). Although the transcriptional organization of this gene cluster in Sulfolobus requires further investigation, it seems to be clear that, in contrast to the Euryarchaeota, the L11 gene is cotranscribed together with the L1, L10, and L12 genes, possibly as part of a long transcript.

**Evidence for the existence of different systems controlling ribosomal protein L1 synthesis in different Archaea:** Our data suggest that at least three different mechanisms of controlling the synthesis of L1 and the cotranscribed ribosomal proteins exist in different Archaea (summarized in Figure 8). Only the regulation of the MvaL1 operon (encoding ribosomal proteins MvaL1, MvaL10, and MvaL12) of the mesophilic M. vannii was investigated in some detail (Hanner et al. 1994; Mayer et al. 1998). MvaL1 is the autoregulator of its operon, which can bind to a specific site on the 23S rRNA or, with a 20-fold lower affinity, to its own mRNA, thereby inhibiting translation of all three cistrons of the operon. So far, we do not yet understand the exact mechanism of MvaL1-mediated regulation. It was demonstrated that autoregulation of MvaL1 occurs before or at the formation of the first peptide bond. Toeprint experiments revealed that MvaL1 does not inhibit the formation of a functional ternary initiation complex (C. Mayer, P. Romby, A. Lingenhel, C. Ehresmann and B. Ehresmann, unpublished results). These data suggest a novel mechanism of translational inhibition that is different from the displacement or entrapment model described for the regulation of ribosomal proteins in E. coli (Philippe et al. 1993; Springer 1996).

From the filter-binding data and the in vitro regulation studies we conclude that the mechanism of translational regulation is similar in mesophilic and thermophilic Methanococci. The difference of more than one order of magnitude observed between the apparent dissociation constants of MjaL1 and MthL1 to their own mRNA and 23S rRNA (Table 2) is a prerequisite for a feedback inhibition based on direct competition between the two binding sites (Zengel and Lindahl 1994). In general, the affinity of L1 proteins from thermophilic Methanococcus species and Sulfolobus to the 23S rRNA is more than 10-fold higher than that of MvaL1 (Table 2). This
strong RNA-protein interaction in thermophilic Archaea, which has also been observed in Thermus thermophilus, a thermophilic bacterium (Köhrer et al. 1998), might contribute to the thermal stability of the ribosome (Cammarano et al. 1983). The fact that MjaL1 and MthL1, compared to MvaL1, exhibit a much stronger inhibitory effect on the L1 synthesis in vitro is probably the result of the stronger mRNA-protein interaction. For the moment we cannot explain why MjaL1 and MthL1 bind to the mutated (M5) mRNA in a highly specific manner, whereas MvaL1 does not bind at all. M5 was constructed as a nonregulatory mutant on the basis of the mutational analysis of the corresponding regulatory site in E. coli (Thomas and Nomura 1987) and the mutational analysis of the L1 binding site of 23S rRNA in E. coli (Said et al. 1988). In E. coli the corresponding mutations completely abolish regulation by L1 and binding of L1 to 23S rRNA, respectively. It remains to be examined whether in thermophilic Methanococcus translation of the L10 and L12 genes is coupled to that of the L1 gene as shown in M. vannielii (Mayer et al. 1998).

It is tempting to assume that in P. horikoshii as well as in M. b. thermolithotrophicum a regulatory mechanism for L1 (and the L1 operon) exists that is similar or identical to that described for Methanococcus. This assumption is based on the similarity of the potential binding sites on mRNA (Figure 4, A and B) to the experimentally confirmed regulatory L1 binding site of Methanococcus. Furthermore, these sites are located 34 and 25 nucleotides, respectively, downstream of the AUG start codon.

In contrast, the potential regulatory AfuL1 binding site discovered in A. fulgidus (Figure 4C) is located in a sequence of the untranslated leader of the AfuL1 gene that would be in direct contact with the ribosome as part of the translation initiation complex. Similar putative regulatory L1 target sites have been suggested for H. cutirubrum (Figure 4D; Shimmin and Dennis 1989), Halofax volcanii, and Haloarcula marismortui (Shimmin and Dennis 1996), located upstream of the start codon at the same distance as the potential AfuL1 binding site.

One could imagine that simple competition between L1 binding and ribosome binding to the mRNA is responsible for translational regulation. In the presence of sufficiently high L1 concentrations, the ribosome could not bind to the mRNA. A competition between

Figure 7.—Alignment of the N-terminal amino acid sequences of archaeal rproteins L1. Identical or conserved residues are shaded. N-terminal methionine residues are boxed. N-terminal residues resulting from the potential use of an alternative, in-frame initiation codon are given in italics.

HCU, H. cutirubrum (as representative of the halophilic Archaea); AFU, A. fulgidus; MBT, M. b. thermolithotrophicum; MJA, M. jannaschii; MTH, M. thermosthrotrophicum; MVA, M. vannielii; PHO, P. horikoshii; SAC, S. acidocaldarius; SSO, S. solfataricus.
the regulatory protein L1 and the ribosome as the basis of translational regulation has been suggested for E. coli, where the regulatory L1 binding site is located in the untranslated leader of the L11 gene, although binding of L1 to this region of the leader has not been directly demonstrated (Zengel and Lindahl 1994).

Our filter-binding experiments demonstrate that Sulfolobus L1 does not bind to a previously suggested structure within the L11 gene. This result is not too surprising, as the potential L1 binding site lacks several of the nucleotides universally conserved in L1 binding sites (Figure 5). So far we have no idea how the synthesis of ribosomal protein L1, or other ribosomal proteins, is controlled in Sulfolobus. It could be regulation at the level of transcription or translation.

We emphasize that our proposals of mechanisms controlling the synthesis of ribosomal protein L1 (operons) in M. b. thermoautotrophicum, P. horikoshii, A. fulgidus, and halophilic Archaea are highly speculative. Further work is required to investigate whether the L1 proteins from these organisms act as translational repressors as suggested.

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