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 *Methanobacterium thermoautotrophicum* 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°; Huber *et al.* 1982) and hyperthermophilic species such as *M. jannaschii* (optimal growth temperature 85°; Jones *et al.* 1983). In the *MthL1*

Corresponding author: Wolfgang Piendl, Institute of Medical Chemistry and Biochemistry, University of Innsbruck, Fritz-Pregl-Strasse 3, A-6020 Innsbruck, Austria. E-mail: wolfgang.piendl@uibk.ac.at

sequence (GenBank accession no. AF044919) and in the *MjaL1* sequence (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 Euryarchaeota *Methanobacterium thermoautotrophicum* (Smith *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, L11, L1, 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(*lac-proAB*) *supE thi r*$^-$ *recA* [F′ *traD36 pro A*$^+$*B*$^+$ *lacI*^{q} *lacZ*Δ*M15*]) (Gibson 1984) was used for the propagation of plasmids. *E. coli* BL21(DE3) [F$^-$ *hsdS gal ompT* (λ*cI857 ind1 Sam7 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 (Jarsch *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 *Nde*I site (including the ATG start codon) and a *Bam*HI 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 pBluescript clones 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. vannielii* 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 pMvaL1.35M5 (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 (Calderone *et al.* 1996), the *E. coli* host was cotransformed with pUBS520, a plasmid that carries the gene for the rare $tRNA^{Arg}_{AGA/AGG}$ (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 μCi of l-[^{35}S]methionine (1200 Ci/mmol; 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; Bioprofil).

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 ^{32}P-labeled RNA fragments were synthesized *in vitro* from templates linearized with *Hin*dIII in the presence of [α-^{32}P]UTP (800 Ci/mmol; New England Nuclear Corp.) 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 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 $MgCl_2$, 500 mm KCl, 1 mm 2-mercaptoethanol, and 0.04% BSA.

RNA structure prediction: RNA secondary structure predictions were performed using two different computer packages, RNAdraw (Matzura and Wennborg 1996) and STAR (Abrahams *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 Batenburg *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: Afu (*A. fulgidus*), Mja (*M. jannaschii*), Mth (*M. thermolithotrophicus*), Mva (*M. vannielii*), 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

	Description / application	Reference
Vectors		
pET11a	Ap^r, T7 promoter and terminator	Dubendorff and Studier (1991)
pBluescript KS / SK	Ap^r, *lac′ IPOZ′* α, T7 promoter	Short *et al.* (1988)
pUC18 / 19	Ap^r, *lac′ IPOZ′* α	Yanisch-Perron *et al.* (1985)
Recombinant plasmids		
pET11a constructs		
pMjaL1.4	Overproduction of MjaL1	Köhrer *et al.* (1998)
pMthL1.4	Overproduction of MthL1	Köhrer *et al.* (1998)
pMvaL1.55M5	Overproduction of MvaL1	Mayer *et al.* (1998)
pSacL1.4	Insert: 0.7-kb *Nde*I-*Bam*HI PCR fragment (template: genomic DNA of *S. acidocaldarius*), containing the *SacL1* gene; overproduction of SacL1	This study
pSsoL1.4	Overproduction of SsoL1	Köhrer *et al.* (1998)
pBluescript expression constructs		
pMjaL1.3	Insert: 0.8-kb *Ssp*I-*Afl*III fragment containing the *MjaL1* gene; *in vitro* synthesis of MjaL1 (regulation studies)	This study
pMjaL1.3M5	Derivative of pMjaL1.3 with M5 mutation	This study
pMvaL1.35	*In vitro* synthesis of MvaL1 (regulation studies)	Hanner *et al.* (1994)
pMvaL1.35M5	Derivative of pMvaL1.35 with M5 mutation	Mayer *et al.* (1998)
pBluescript transcription constructs		
pMthL1.23S	Insert: 0.15-kb *Sac*I-*Hin*dIII 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	This study
pMvL1.23S	*In vitro* synthesis of a 210-nt-long *M. vannielii* 23S rRNA fragment containing the MvaL1 binding site	Mayer *et al.* (1998)
pSacL1.23S	Insert: 0.16-kb *Sac*I-*Hin*dIII 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	This study
pMthL1.Tk	Insert: 0.25-kb *Sac*I-*Hin*dIII 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	This study
pMthM5.Tk	Derivative of pMthL1.Tk with M5 mutation	This study
pMvL1L10	*In vitro* synthesis of a 250-nt-long *M. vannielii* mRNA fragment containing the MvaL1 binding site	Mayer *et al.* (1998)
pMvM5L10	Derivative of pMvL1L10 with M5 mutation	Mayer *et al.* (1998)
pSacL11.Tk	Insert: 0.25-kb *Sac*I-*Hin*dIII 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	This study
pMva16S-S8BS.3	*In vitro* synthesis of a 220-nt-long *M. vannielii* 16S rRNA fragment containing the MvaS8 binding site	Köhrer *et al.* (1998)

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. vannielii* with rather high affinity (Köhrer *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. vannielii* (Figure 1A). The affinity of MjaL1 and MthL1 for their own mRNA was compared to that for their own 23S rRNA. To minimize potential nonspecific interactions, a binding buffer containing 500 mm KCl was chosen (Köhrer *et al.* 1998). A 250-nucleotide transcript encompassing the MthL1 mRNA-binding site

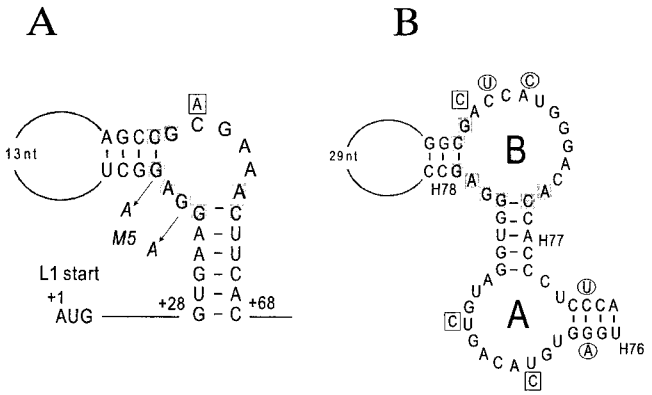

Figure 1.—Comparison of (A) mRNA and (B) 23S rRNA binding sites for ribosomal protein L1 of different Methanococcus species. The structures of *M. thermolithotrophicus* are shown. Nucleotides different in *M. jannaschii* are boxed, nucleotides different in *M. vannielii* are circled. Arrows indicate 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.

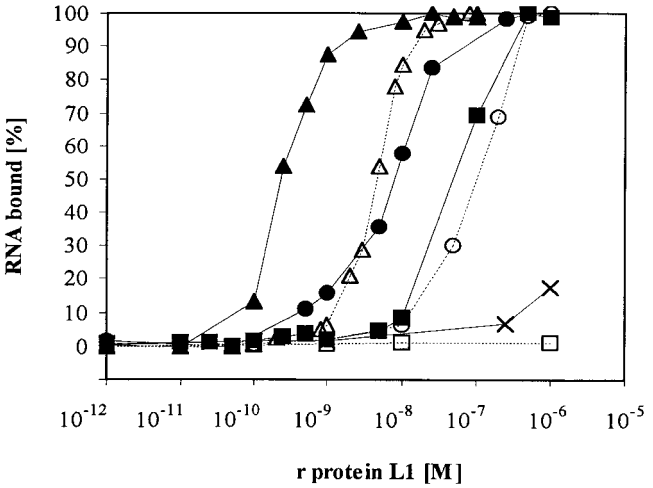

Figure 2.—Binding curves for the interaction of ribosomal proteins MthL1 (solid lines, solid symbols) and, for comparison, MvaL1 (broken lines, open symbols) with their homologous 23S rRNA (▲, △), wild-type mRNA (●, ○), and mutated M5mRNA (■, □) binding sites. A 16S rRNA fragment (solid line, X) was 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.

(synthesized from template pMthL1.Tk), the corresponding fragment encompassing the M5 mutation thereof, and a 150-nucleotide fragment containing the 23S rRNA binding site from *M. thermolithotrophicus* (synthesized 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 interaction of MjaL1, MthL1, and MvaL1 with their homologous rRNA and mRNA fragments are summarized in Table 2. The apparent dissociation constants of the homologous MjaL1-rRNA complex (K_d: 1.2×10^{-10} M) and the MthL1-rRNA complex (K_d: 3.1×10^{-10} M) are virtually identical and in good agreement with the dissociation constants determined for the complexes of the same proteins with heterologous 23S rRNA fragments derived from *M. vannielii* (Köhrer *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 species. In contrast to MvaL1, MjaL1 and MthL1 do bind to their specific mRNA target site carrying the nonregulatory M5 mutant in a highly specific manner, as compared to the noncognate 16S rRNA fragment (Figure 2; Table 2).

Ribosomal L1 proteins from thermophilic Methanococcus 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 thermophilic ribosomal proteins. Plasmids 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 translational 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

Binding of ribosomal protein L1 from the thermophilic and mesophilic Methanococcus species to their homologous 23S rRNA and mRNA

Protein	RNA	K_d (M)	Saturation level (%)
MjaL1	23S rRNA	1.2×10^{-10}	60–90
	mRNA-WT	3.9×10^{-9}	36–40
	mRNA-M5 mutant	2.5×10^{-7}	67
MthL1	23S rRNA	3.1×10^{-10}	60–65
	mRNA-WT	7.2×10^{-9}	60–70
	mRNA-M5 mutant	3×10^{-8}	80–90
MvaL1	23S rRNA	5×10^{-9}	39–65
	mRNA-WT	1.5×10^{-7}	52–58
	mRNA-M5 mutant	—	—

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 *Mb. thermoautotrophicum* 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 *Mb. thermoautotrophicum* 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 pSacL11.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-

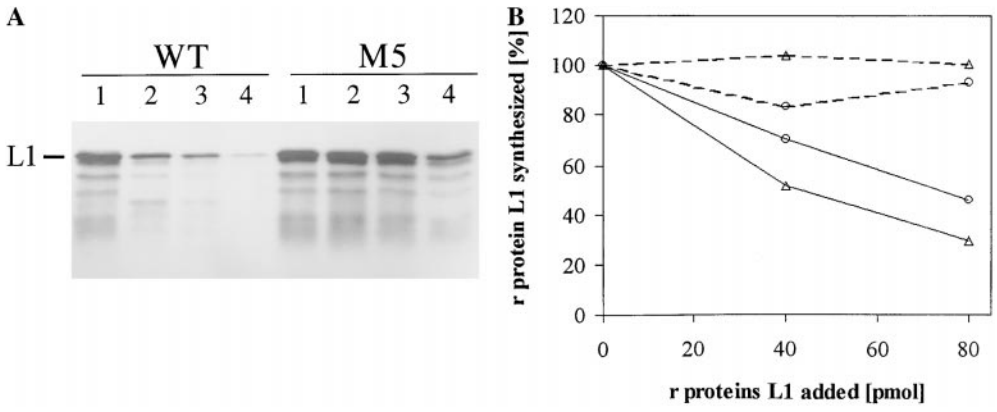

Figure 3.—Effects of MjaL1 and, for comparison, of MvaL1 on the DNA-directed *in vitro* synthesis of MvaL1. (A) Autoradiogram of an 18% gel with 1-[^{35}S]methionine-labeled proteins synthesized from the wild-type plasmid pMvaL1.35 and the nonregulatory mutant pMvaL1.35M5, respectively. Lane 1, control, no MjaL1 added; lanes 2–4, addition of 40, 80, and 120 pmol of MjaL1 to the reaction mixture. Samples (10 μl) were loaded on the gel. (B) Graph of the scanning data showing the inhibition of synthesis of MvaL1 synthesized from the wild-type template pMvaL1.35 (solid lines) and mutant template pMvaL1.35M5 (broken lines) by MjaL1 (△) and MvaL1 (○). The numbers are given as percentages; controls with no addition of L1 proteins are 100%.

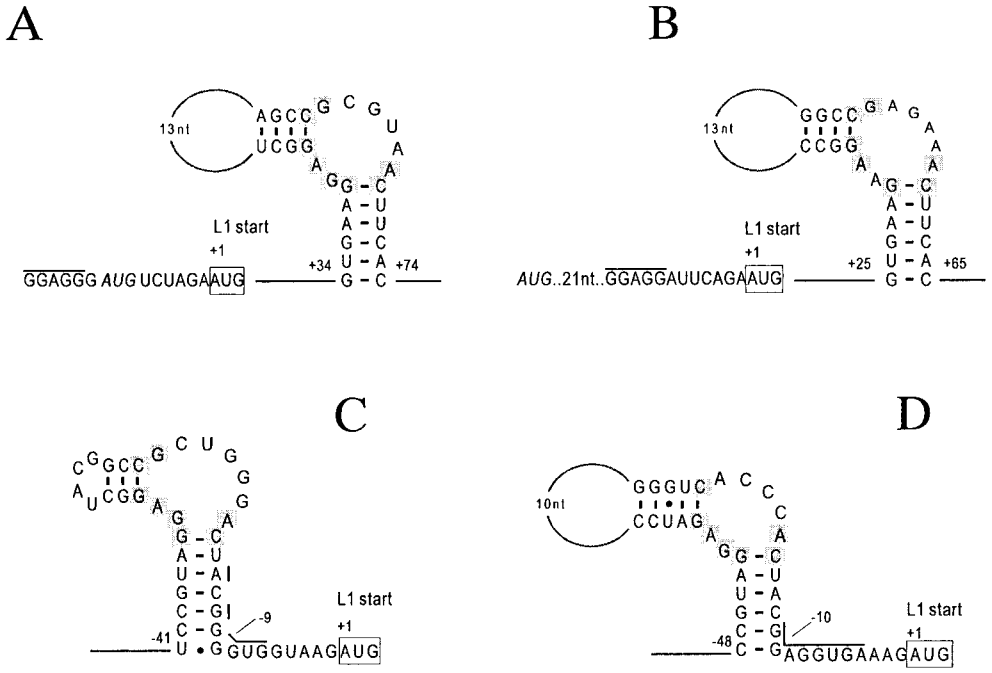

Figure 4.—Secondary structures of putative L1 binding sites on their own mRNA from (A) *P. horikoshii*, (B) *Mb. thermoautotrophicum*, (C) *A. fulgidus*, and (D) *H. cutirubrum*. Base numberings are taken from the translation start. Highly conserved nucleotides are shaded. Potential translation initiation sequences complementary to the 3′ end of 16S rRNA are overlined. Start codons are boxed. Alternative, in-frame start codons suggested by other authors are given in italics (see discussion). The Halobacterium structure is taken from Shimmin and Dennis (1989).

ent dissociation constant of the SacL1/23S rRNA complex is 5.3×10^{-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: 1×10^{-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*, *Mb. 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*, *Mb. 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 *Mb. 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. acidocaldarius*, a Shine-Dalgarno sequence overlapping with the start codon has been reported (Ramirez *et al.* 1994). It is difficult to

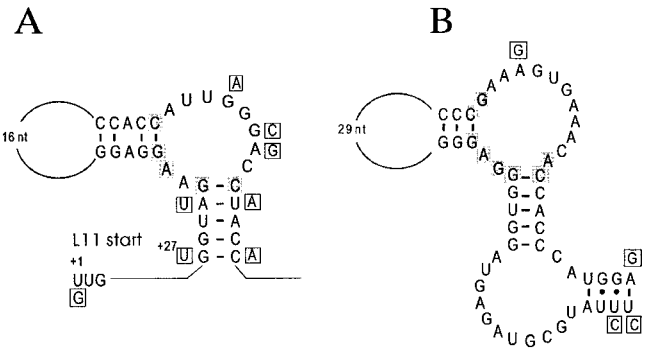

Figure 5.—Secondary structure of a putative L1 binding site located in the L11 mRNA (A) and, for comparison, of the L1 binding site on the 23S rRNA (B) from *S. acidocaldarius*. Both structures are taken from Ramirez *et al.* (1994). Highly conserved nucleotides are shaded. Nucleotides different in *S. solfataricus* are boxed. Base numberings in A are taken from the L11 translation start.

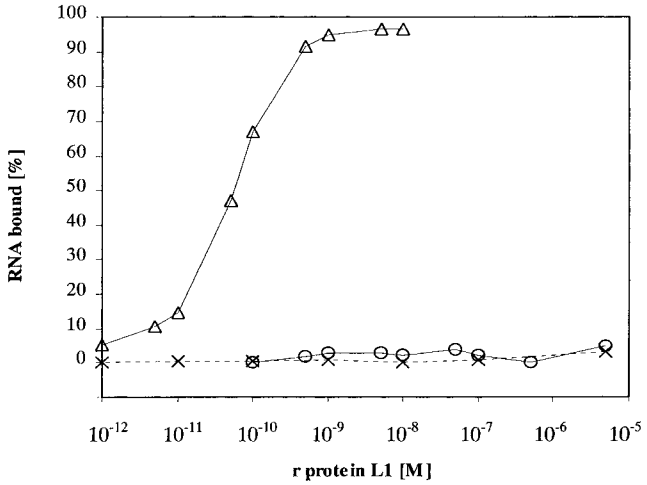

Figure 6.—Binding curve for the interaction of ribosomal protein SacL1 with the homologous 23S rRNA (△, solid line), the proposed binding site on the L11 mRNA (○, solid line) and, as a control, a 16S rRNA fragment (X, broken line). A representative experiment is shown. Values of RNA retained on the filter in the absence of protein (2–5%) were subtracted before the data were blotted.

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 archaeal ribosomal *L1* genes: The order of the genes for ribosomal proteins is highly conserved in Bacteria and Archaea (Wächtershäuser 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. vannielii* (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 *Mb. thermoautotrophicum* 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 AAAAGTTTAAA is located 60 bp upstream of the initiation codon, which fulfills the criteria of a good transcription initiation site (Suckow *et al.* 1998). In *Mb. thermoautotrophicum*, 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-L1-L10-L12 tetracistronic transcript (Ramirez *et al.* 1994). Thus the promoter for this tetrameric 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. vannielii* 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

```
HCU-L1  - - - - - - - - - - - - - - - - A D N D - I E E A V A R A L E D A - P Q - R N F
AFU-L1  - - - - - - - - - - - - - - - -[M]I V D K D K L V K S I E E A I K N G K K - - R R F
MBT-L1  - M N L F F E V H G G F R[M]Q Q E - I M E A V K K A K E L S R P - - R N F
MJA-L1  M L F V K Y K K E R R M T[M]D R E A L L Q A V K E A R E L A K P - - R N F
MTH-L1  - - - - - - - - - - - - - - - -[M]D R E N I L K A V K E A R S L A K P - - R N F
MVA-L1  - - - - - - - - - - - - - - - -[M]D S A Q I Q K A V K E A R T L A K P - - R N F
PHO-L1  - - - - - - - - - M S R[M]A F D R Q K I V E A V K E A K A R A K P - - R N F
SAC-L1  - - - - - - - - - M K K[M]L A D K E S L I E A L K L A L S T E Y N V K R N F
SSO-L1  - - - - - - - - - - - -[M]Q I V D R S N L E A S L K L A L S P E N N P K R N F
```

Figure 7.—Alignment of the N-terminal amino acid sequences of archaeal r proteins 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, *Mb. thermoautotrophicum*; MJA, *M. jannaschii*; MTH, *M. thermolithotrophicus*; MVA, *M. vannielii*; PHO, *P. horikoshii*; SAC, *S. acidocaldarius*; SSO, *S. solfataricus*.

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 Methanococci translation of the *L10* and *L12* genes is coupled to that of the *L1* gene as shown in *M. vannielii* (Mayer *et al.* 1998).

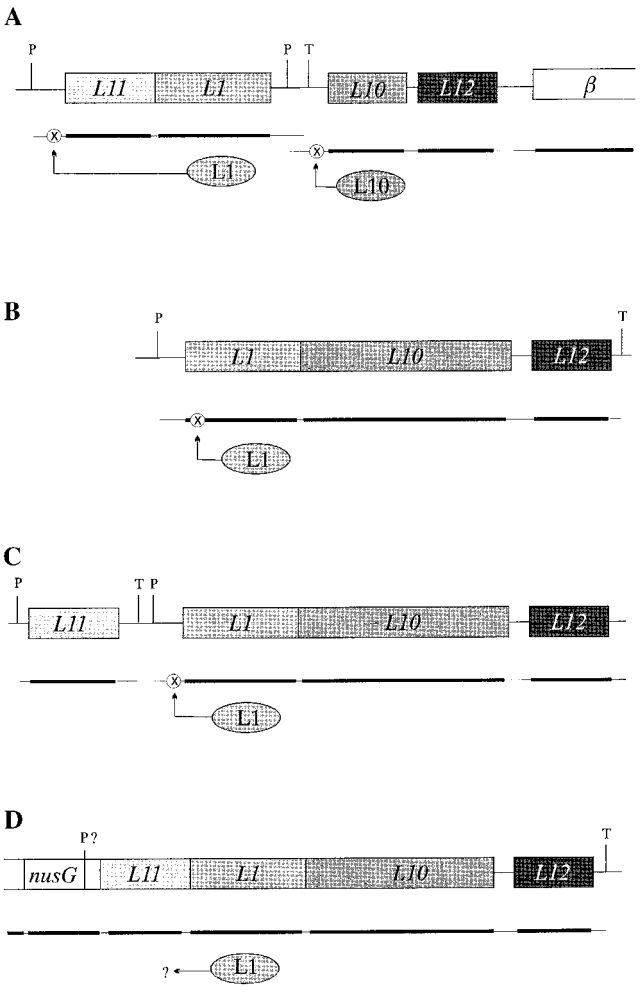

Figure 8.—Transcriptional organization and regulation of the L11, L1, L10, and L12 encoding region from (A) *E. coli*, (B) Methanococcus, (C) halophilic Archaea and *A. fulgidus* and (D) Sulfolobus. Genes are shown as boxes. The localization of the regulatory target sites on the mRNA is indicated (⊗). In the Bacterium *E. coli*, which is shown for comparison, the region is split into two regulatory units (A). In *A. fulgidus* the *L11* gene is replaced by the gene for the subunit α of an oxidoreductase (C). P, promoter; T, terminator.

It is tempting to assume that in *P. horikoshii* as well as in *Mb. thermoautotrophicum* 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), *Haloferax volcanii*, and *Haloarcula marismortii* (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

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 *Mb. 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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