Structure, organization and evolution of the L1 equivalent ribosomal protein gene of the archaebacterium *Methanococcus vannielii*

G.Baier, W.Piendl*, B.Redl and G.Stöffler
Institut für Mikrobiologie der Medizinischen Fakultät, Universität Innsbruck, Fritz-Pregl-Straße 3, A-6020 Innsbruck, Austria

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ABSTRACT

The gene for ribosomal protein MvaL1 from the archaebacterium *Methanococcus vannielii* was cloned and characterized. It is clustered together with the genes for MvaL10 and MvaL12, thus is organized in the same order as in *E.coli* and other archaebacteria. Unexpectedly, analysis of the sequence in front of the MvaL1 gene revealed an ORF of unknown identity, whereas in *E.coli*, *Halobacterium* and *Sulfolobus solfataricus* the gene for the L11 equivalent protein is located in this position. Northern blot analysis revealed a single tricistronic transcript encoding proteins MvaL1, MvaL10 and MvaL12. The 5'-end of the MvaL1-L10-L12 transcript contains a region that has a sequence and structure almost identical to a region on the 23S rRNA which is the putative binding domain for MvaL1, and is highly similar to the *E.coli* L11-L1 mRNA leader sequence that has been implicated in autogenous translational regulation. Amino acid sequence comparison revealed that MvaL1 shares 30.5% identity with ribosomal protein L1 from *E.coli* and 41.5% and 33.3% identity with the L1-equivalent proteins from the archaebacteria *H.cutirubrum* and *S.solfataricus* respectively.

INTRODUCTION

The structure and function of the ribosome has been conserved throughout evolution (1,2). Acccordingly, the essential features of r-proteins forming ribosomal domains are expected to be conserved, although having been subject to evolutionary tinkering to refine efficiency and accuracy of protein synthesis. On this account it could be shown by chimeric reconstitution experiments, that r-protein MvaL1 of the methanogenic archaebacterium *Methanococcus vannielii* is the structural counterpart to *E.coli* r-protein L1, even being able to functionally replace the *E.coli* r-protein in vitro (3).

In *E.coli* the gene for L1 is linked with the genes encoding L11, L10, L12 and two genes encoding RNA polymerase subunits (β and β') in the order L11-L1-L10-L12-β-β' (4). Recently, the organization of the genes encoding the L11, L1, L10 and L12 equivalent r-proteins from the halophilic archaebacteria *Halobacterium halobium*, *Halobacterium cutirubrum* and the sulfur-dependent thermophilic archaebacterium *Sulfolobus solfataricus* has been characterized. These r-protein genes are clustered in the same order as that in *E.coli*, however the transcriptional units are different (5,6,7). A. Böck and his co-workers characterized two ribosomal gene clusters from *M.vannielii* corresponding to the *E.coli* spc- and str-operon (8,9,10). Their results indicate that the gene organization is almost identical to that of the eubacterial operons whereas the primary structure of the r-proteins is more eukaryotic in nature. This is also true for the L10 and L12 equivalent proteins from *M.vannielii* which have been already examined (11,12).

In order to further investigate the structure, organization and evolution of r-protein genes in methanogenic archaebacteria, we have cloned and characterized the gene encoding the L1 eqivalent r-protein from *M.vannielii* together with the flanking regions. Additionally we have investigated the transcriptional organization and discuss the putative implication of MvaL1 in autogenous translational regulation similar to *E.coli* r-protein L1. Amino acid alignments are given, in order to elucidate the evolutionary relationships between the archaebacterial proteins and their eubacterial equivalent.

MATERIALS AND METHODS

Strains and plasmids

Cells from *M.vannielii* DSM 1224 were kindly provided by A. Böck. *E.coli* r-protein L1-deficient mutants AM1422 and MV17−10 were supplied by E. Dabbs (13). *E.coli* strain TG2 (δlac−pro, supE, thi-, r-, recA,F'tra D36, pro A^+B^+,lac I^q lac ZδM15) (14) was used as host for transformations. Vectors used were M13mp18, M13mp19, puC18 and puC19 (15).

Recombinant DNA techniques

Chromosomal DNA from *M.vannielii* was isolated as described earlier (16). Restriction endonucleases and DNA modifying enzymes were obtained from Boehringer (Mannheim, FRG) and were used as described by the manufacturer. Agarose gel electrophoresis, preparation and purification of plasmid DNA,

* To whom correspondence should be addressed

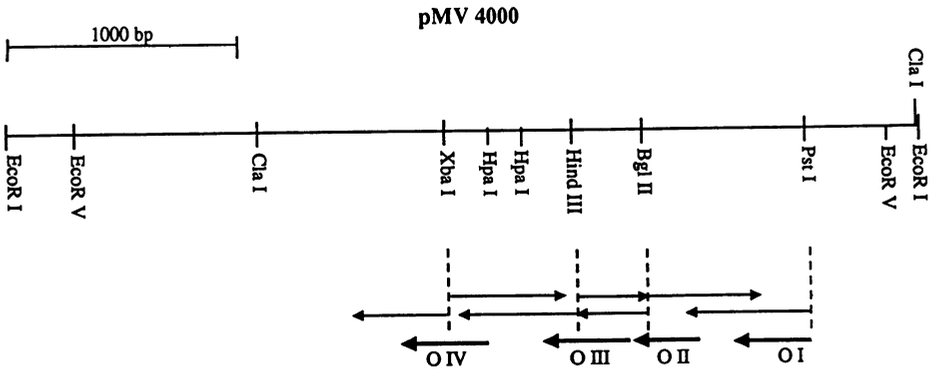

Fig.1: Restriction map of pMV4000 and sequencing strategy for the fragment containing the MvaL1 gene. Bars at the bottom of the figure represent M13 clones used for sequencing. OI, OII, OIII and OIV were synthetic oligonucleotides used as primers for plasmid sequencing of pMV4000.

ligation, procedures for radiolabelling DNA were carried out using established procedures (17). DNA fragments were isolated from agarose gels as described (18). Using an end labelled oligonucleotide specific for MvaL1 as a probe the hybridization conditions for Southern blots and colony transfers on nylon membranes (Hybond N,Amersham) were 35°C, 6×SSC, 0.1% SDS, 10×Denhardt solution and 100 μg/ml tRNA. Filters were washed in 1×SSC, 0.1% SDS at 43°C for 5 min. Transformation of E.coli TG2 and the L1-deficient E.coli mutants AM1422 and MV17-10 was done as described elsewhere (19).

DNA sequence determination

DNA sequence analysis was performed by the dideoxy chain termination method using single stranded M13-DNA (20) or double stranded DNA cloned in pUC19 (21). DNA sequence comparisons were performed with the programs Gap, Bestfit and Align and the MIPSX data bank (Martinsried, FRG) on a Microvax A computer system.

RNA preparation and analysis

M. vannielii cells were grown to the mid-log phase and harvested; total RNA was prepared according to (22). The RNA was separated on a 1% formamide-agarose gel as described (23), transferred to a nylon membrane and hybridized to a nick translated DNA probe at 42°C in a buffer containing 6×SSC, 10×Denhardt solution, 0.1% SDS, 50% (v/v) formamide and 100 μg/ml tRNA. Filters were washed at 50°C in 2×SSC, 0.1% SDS.

To define the transcriptional initiation site, mung bean nuclease mapping (24) and primer extension (25) were performed as described.

Immunoblotting analysis

Cell extracts of *E.coli* harbouring plasmid pMV4000 or pMvaL1 were separated by SDS gel electrophoresis in 17.5% polyacrylamide gels and the proteins were electroblotted to nitrocellulose (26). Blots were incubated with an antiserum raised against the purified MvaL1 and immunostaining was performed using anti-goat IgG alkaline phosphatase conjugate as secondary antibody and 5-bromo-4-chloro-3-indolylphosphate as substrate for the phosphatase (27).

Nomenclature

Originally proteins of the large ribosomal subunit from *M. vannielii* had been named ML1, ML2, etc. according to their position on two-dimensional electropherograms (28). In those cases where unambiguous homology to the r-proteins of *E.coli* was later found by sequence analysis, the r-proteins were renamed in order to express this homology. For example, r-protein ML6 thus became MvaL1 (this paper), ML2 became MvaL10 (11) and ML8 was renamed MvaL12 (12).

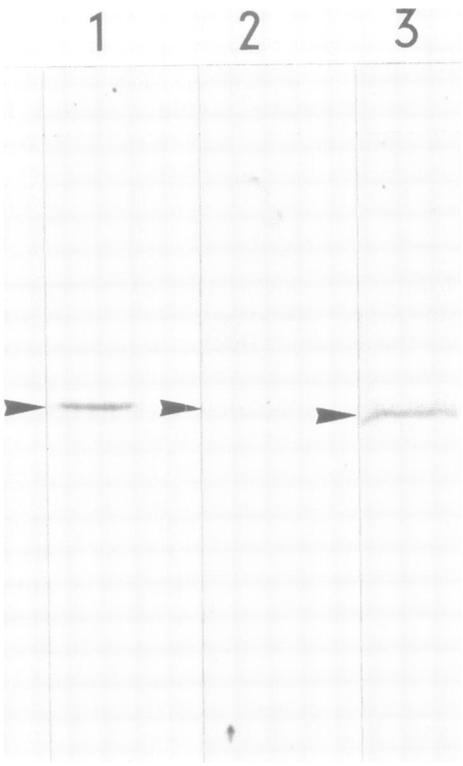

Fig.2: Western blot analysis of cell extracts from *E.coli* harbouring pMvaL1. Blots were incubated with an antiserum specific for MvaL1. Lane 1: 500 ng of authentic protein MvaL1, lane 2: cell extract of *E.coli* TG2 harbouring pUC18, lane 3: cell extract of *E.coli* TG2 harbouring pMvaL1.

RESULTS AND DISCUSSION

Cloning and identification of the gene for MvaL1

Before setting up the cloning experiments we had to consider that overproduction of r-protein L1 in *E.coli* causes a specific decrease in L11 synthesis, resulting in a drastic inhibition of cell

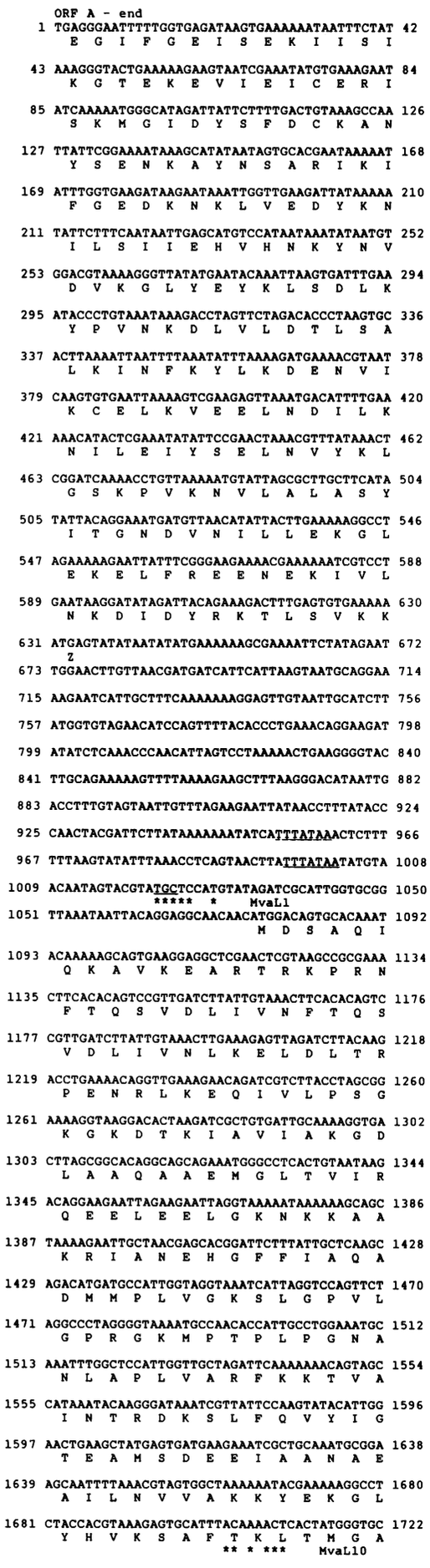

Fig.3: The DNA and deduced amino acid sequence of the MvaL1 gene and its flanking regions. Sequences resembling the archaebacterial consensus promotor are underlined and putative ribosome binding sites upstream of the protein start points are marked by asterisks.

growth (29). As r-protein MvaL1 is the structural and functional counterpart to *E.coli* r-protein L1 (3), we could not exclude the possibility that overexpression of a cloned MvaL1 gene in *E.coli* might affect growth of the host. Therefore for all cloning and subcloning experiments plasmids pUC18/19 were chosen as vectors, since the expression of cloned foreign DNA fragments can be regulated via the lac-promotor. Archaebacterial promotors are not recognized in *E.coli* (30).

According to the codon usage in *Methanococcus* (GC-content 31,1 mol%) a 17-mer oligonucleotide 5'AC(T/A)GC-TTTTTG(A/T)ATTTG3' complementary to the N-terminal amino acid sequence (positions 5−10: QIQKAV) was synthesized, endlabelled and used to probe restriction enzyme digests of *M.vannielii* genomic DNA. A 4 kb EcoRI fragment, which hybridized to the oligonucleotide was chosen for cloning (data not shown).

Size fractionated 3.5−4.5 kb EcoRI fragments of *Methanococcus* genomic DNA were cloned into plasmid pUC19 and transformed into *E.coli* TG2. Using the same oligonucleotide mix as a probe 8 positive clones were detected among 2500 transformants. All were shown to contain the same insert and one was chosen as pMV4000. A detailed restriction map of this 4 kb EcoRI fragment is shown in Fig.1.

In order to confirm that we had cloned the MvaL1 gene, the gene products of pMV4000 and of pMvaL1, a subclone containing the 5'HindIII-3'PstI fragment which hybridized to the MvaL1 specific oligonucleotide probe, was analyzed by immunoblotting using an antiserum raised against the purified protein MvaL1. For these experiments, transformants were grown in the presence of IPTG to achieve maximal expression of the cloned gene.

We could demonstrate that MvaL1 was expressed in *E.coli* harbouring pMvaL1 (Fig.2) but not in those harbouring pMV4000. The M_r of the recombinant MvaL1 was equal to that of MvaL1 purified from *M.vannielii*, namely 22.2 kD as determined by SDS-PAGE. By preabsorption of the MvaL1 specific antiserum with purified MvaL1 this immunoreaction was obviated, proving the immunological identity of purified MvaL1 with the recombinant translational product of pMvaL1.

Next pMvaL1 was transformed into *E.coli* mutants AM1422 and MV17-10 lacking r-protein L1. The generation time of these mutant strains is about twice that of the parental wildtype strain (31). After induction with IPTG both mutants, AM1422 and MV17-10 harbouring pMvaL1 had their growth rate fully restored to wild type level (to be published elsewhere).

Nucleotide sequence analysis of the MvaL1 gene and its flanking regions

The sequencing strategy of the region which contains the MvaL1 gene is shown in Fig. 1 and the results of the sequence analysis

are detailed in Fig.3. We found an ORF coding for a protein of 222 amino acids. As this ORF contained an N-terminal amino acid sequence identical to the one determined by protein sequencing of the purified MvaL1, it could be identified as the gene encoding MvaL1.

Downstream of the MvaL1 gene, the N-terminus of another ORF was found, which could be identified, by comparison of the deduced amino acid sequence with the registered proteins of the EMBL data base, as the N-terminal part of MvaL10. Our cloned 4 kb fragment showed an overlap of 20 bp with pMVaX1 containing the MvaL7/12 gene (12) and the C-terminus of MvaL10. The complete sequence of the MvaL10 gene has been published previously (11).

Upstream of the MvaL1 gene, separated by a spacer region of 441 bp another open reading frame was found.

The gene for MvaL1 is clustered together with those for MvaL10 and MvaL12, known to be equivalent to ribosomal protein L10 and L12 from *E.coli* (11,12), thus the *M.vannielii* genes are organized in the same order as those in *E.coli* and in the archaebacteria *H.halobium*, *H.cutirubrum* and *S.solfataricus*. Unexpectedly, the open reading frame in front of the MvaL1 gene is of unknown identity, whereas in *E.coli* and all archaebacteria investigated so far, the gene for the L11 equivalent protein is located in this position (4,5,7,32).

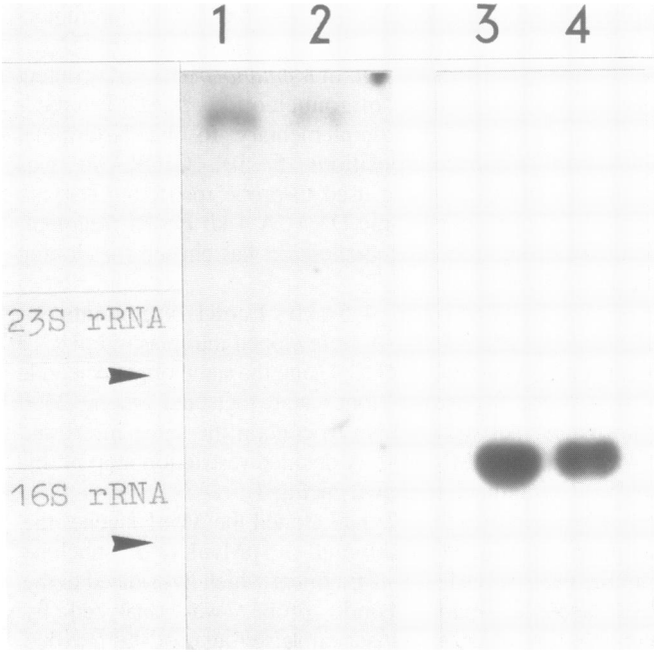

Fig.4: Northern blot analysis of *M.vannielii* RNA. Lane 1 and 2: 20 μg and 10 μg of total RNA hybridized with labelled EcoRI/HindIII restriction fragment from pMV4000. lanes 3 and 4: 20 μg and 10 μg of total RNA hybridized with labelled BglII/EcoRI restriction fragment from pMV4000. The migration position of 16S and 23S rRNA is indicated.

Transcription analysis

Restriction fragments of pMV4000 (shown as solid boxes in Fig.5) were used as probes in Northern blot analysis. We found that the 5'BglII-3'EcoRI fragment hybridized to a transcript with a size of 2.1 kb, whereas the 5'EcoRI-3'HindIII fragment hybridized to a transcript of about 6 kb. No minor transcripts could be detected in Northern blotting, mung bean nuclease protection and primer extension analysis (Fig.4).

These data, summarized in Fig.5 show that the MvaL1, MvaL10 and MvaL12 genes are cotranscribed in *Methanococcus*. This transcription pattern is identical to that found in *H.halobium* and *H.cutirubrum* (5,32), but different from that in *E.coli*, where L1 is cotranscribed together with L11; L10 and L12 are cotranscribed together with β and β' subunits of the RNA polymerase. The identity of the 6 kb transcript remains to be clarified.

To define the transcriptional initiation site, in vivo transcripts from *M.vannielii* were characterized by mung bean nuclease-mapping and by oligonucleotide directed primer extension using reverse transcriptase. For mung bean nuclease-mapping the HindIII-BglII DNA-fragment derived from pMV4000, which extended from position 132 bp in the MvaL1 gene sequence to 220 bp upstream of the ATG codon, was used. For primer extension the 5'-end labelled oligonucleotide 5'ACTGCTTTTTGTATTTG3' was used as primer.

Unexpectedly, both oligonucleotide directed reverse transcription (Fig.6 b) and mung bean nuclease-mapping (Fig.6 a) determined the transcriptional initiation site to be a C, 52 nt upstream of translation initiation codon AUG, although in archaebacteria initiation occurs often at the central G or at the nearby purine nucleotide (30).

Detailed analysis of the DNA sequence in front of the transcription initiation site revealed a similarity to a sequence motif postulated to be the archaebacterial promotor (33,34,35). Two conserved sequences were detected: TTTATA, 22 nucleotides upstream of the transcription initiation site, which is identical to the 'box A' of the archaebacterial consensus

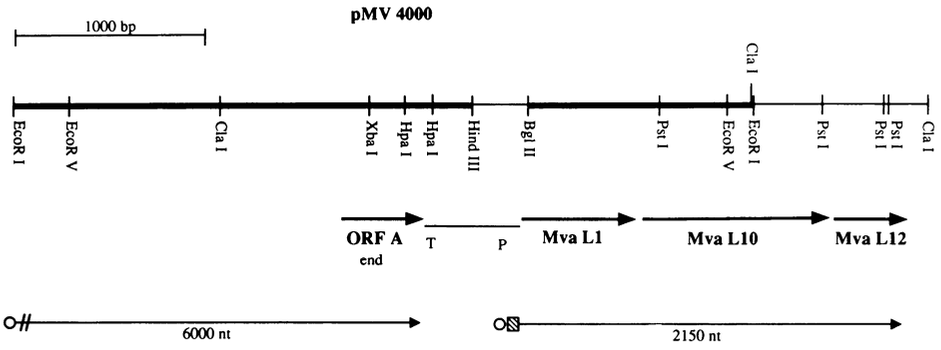

Fig.5: Organization of the genes encoding MvaL1, MvaL10 and MvaL12 and their in vivo transcripts. DNA fragments indicated as solid boxes were used as probes for Northern blot analysis.

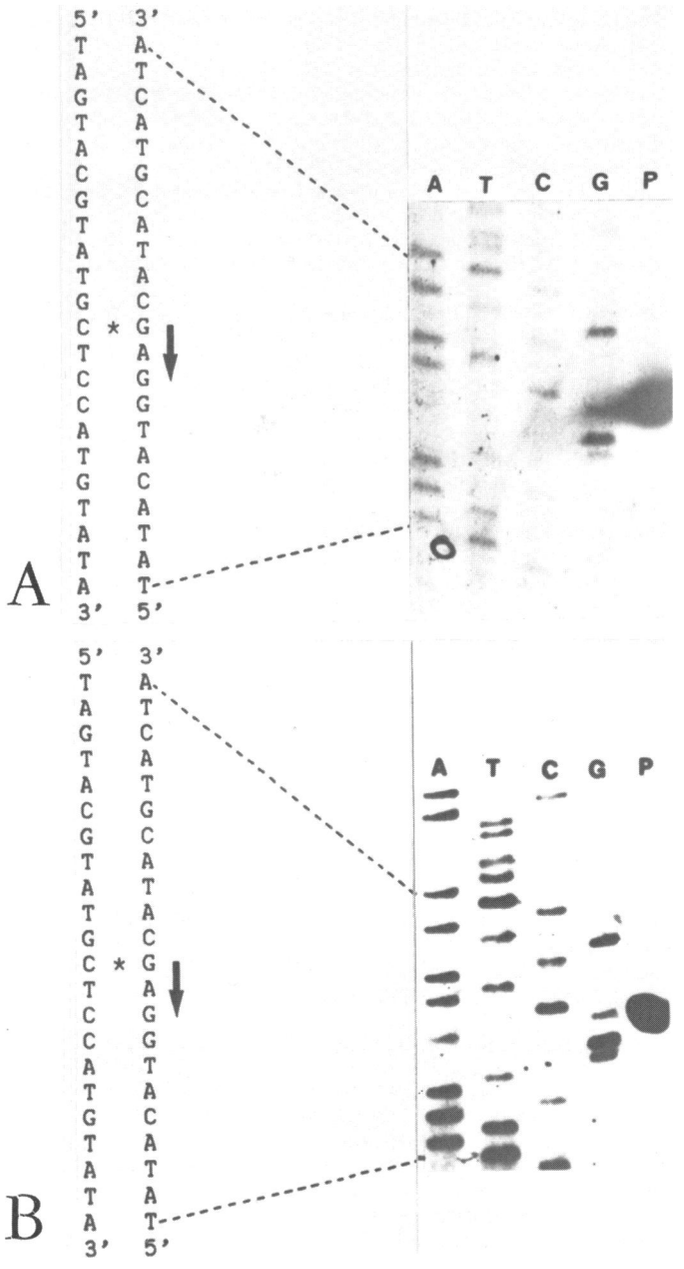

Fig.6: a) Mung bean nuclease-mapping of the 5'-end of the MvaL1-L10-L12 mRNA from *M.vannielii* (P). Lane A,T,C and G are the sequence ladder. The first nucleotide in the protected fragment is indicated (*). b) Primer extension analysis of the transcriptional start site of the MvaL1-L10-L12 mRNA from *M.vannielii* (P). Sequence reactions (A,T,C and G) with the corresponding oligonucleotide as primer were used as exact size marker. The transcript start site is indicated (*).

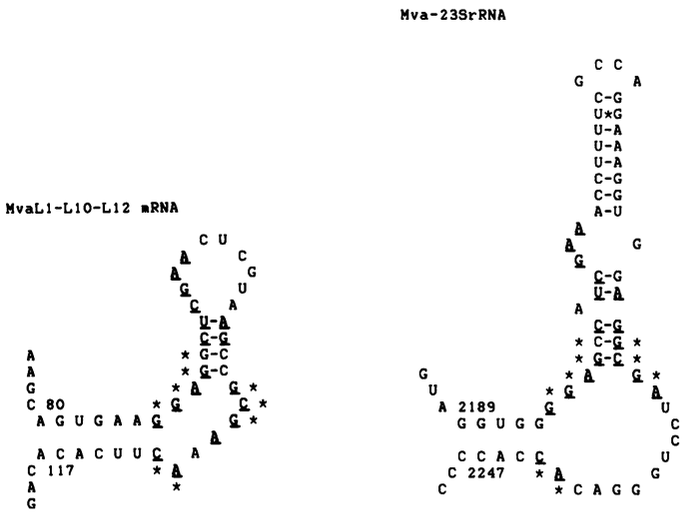

Fig.7: Conserved sequence and structure of the putative binding domain of MvaL1 on 23SrRNA and on the MvaL1-L10-L12 mRNA. Nucleotides conserved in the corresponding binding domains of L1 in *E.coli* are marked by (*). The sequence of the 23SrRNA of *M.vannielii* was taken from (43).

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     1                                                          61
EL1*AKLTKRMRVIREKVDATKQYDINEAIALLKELATAKFVESVDVAVNL-GIDARKSDQNVRG
Mva*MDSAQIQKAVKE-ARTRKPRNFTQSVDLI-----VNFTQSVDLIVNLKELDLTRFENRLKE
Hcu*MADNDIEEAV---------ARALEDAPQ-------RNFRETVDLAVNI.RDLDLNDPSQRVDE
Sso*MKKVLADKESLI-EALKLA--LSTEYNVK------ANFTQSVEIILTFKGIDMKKGDLKLRE

     62                                                        122
EL1*ATVLPHGTGRSVRVAVFTQ-GANAEAAKAAGAELVGMEDLAD--QIKKGEMNF------DVVI
Mva*QIVLPSGKGKDTKIAVIAK-GDLAAQAAEMGLTVIRQEELEELGKNKKAAKRIANEHGFFI
Hcu*GVVLPSGTGQETQIVVFAD-GETAVRADDVADDVLDEDDLSDLADDTDAAKDLADETDFFV
Sso*IVPLPKQPSKAKRVLVVPSFEQLEYAKKASPNVVITREELQKLQGQKRPVKKLAIQNEWFL

     123                                                       183
EL1*ASPDAMRVVGQ-LGQVLGPRGLMPNPKVGYVTPNVAEAVKNAKAGQVRYRNDKNGIIHTTI
Mva*AQADMMPLVGKSLGPVLGPRGKMPTPLPG--NANLAPLVARFKKTVAINTRDKS-LFQVYI
Hcu*AEAPMMQDIVGALGQVLGPRGKMPTPLQP--DDDVVDTVNRMKNTVQIRSRDRR-TFHTRV
Sso*INQESMALAGRILGPALGPRGKFPTPLPN--TADISEYINRFKRSVIVKTKDQP-QVQVFI

     184                                                       243
EL1*GKVDFDADKLKENLEALLVALKKAKPTQAKGVY-IKKVSISTTMGAGVAVDQAGLSASVN
Mva*GTEAMSDEEIAANAEAILNVVAK---KYEKGLYHVKSAFTKLTMGAAAPISK--------
Hcu*GAEDMSAEDIASNIDVIMRRLHA---NLEKGPLNVDSVYVKTTMGPAVEVA---------
Sso*GTEDMKPEDLAENAIAVLNAIEN---K-AKVETNLRNIYVKTTMGKAVKVKRA-------
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Fig.8: Alignment of amino acid sequences of four ribosomal proteins belonging to the L1 family. The alignment was made by the computer program ALIGN. The amino acid sequences of the *E.coli* L1, *H.cutirubrum* and *S.solfataricus* L1 equivalent r-proteins were taken from (4,7). EL = *E.coli*, Mva = *M.vannielii*, Hcu = *H.cutirubrum*, Sso = *S.solfataricus*.

promotor and a sequence ATGC, which constitutes the 'box B' at the site of transcription initiation (Fig.3). A second, identical 'box A' motif was found 64 nt upstream of the transcription initiation site. A similar situation—two 'box A' motifs with identical spacing—has been observed in a promotor region of *H.cutirubrum* (32).

The AT-rich DNA sequence of 'box A' resembles the TATA-boxes found in eukaryotic polymerase II promotors (30).

A putative ribosome-binding site was found 5 nt upstream of the initiation codon AUG (Fig.3).

Putative structures for rRNA binding and autogenous translation regulation

E.coli r-protein L1 is an RNA-binding protein, binding directly and specifically to 23S rRNA (36). The binding site of L1 on *E.coli* 23S rRNA has been characterized (37,38) and it was shown that *E.coli* r-protein binds also to large rRNA's from phylogenetically unrelated species like *Bacillus stearothermophilus* and *Dictyostelium discoideum* (39).

Sequencing data confirm that the primary and secondary structure of the L1 binding region on the 23S/28S rRNA is highly conserved in eubacteria, archaebacteria and eukaryotes (40,41,42,43,44,45, 46).

Ribosomal protein L1 can also bind to a region within the leader sequence on the *E.coli* L11-L1 mRNA to mediate autogenous translation regulation (29,47). This mRNA binding region exhibits significant structural similarity to the binding domain in 23S rRNA (41,48).

A striking structural homology was found between a region

of the 5'-end of the MvaL1-L10-L12 transcript (positions 80−117) and the putative binding domain for the L1 equivalent protein in 23S rRNA (positions 2189−2247) of *M.vannielii* (Fig.7). Furthermore, both the primary and secondary structure of these sites are again highly similar to the *E.coli* L11-L1 mRNA leader sequence.

Within the 74 nt long untranslated leader sequence of the tricistronic L1-L10-L12 transcript of *H.cutirubrum*, a similar region has been detected that has a sequence and structure almost identical to the L1 equivalent binding site in 23S rRNA of *Halobacterium* and that is highly similar to the L1 target site in the *E.coli* L11-L1 mRNA leader sequence (32).

In contrast to *E.coli* and *Halobacterium*, this putative binding site for MvaL1 in the MvaL1-L10-L12 mRNA is not part of an untranslated leader sequence of the mRNA, but is within the MvaL1 coding region. A similar situation is found in the *E.coli* spc-operon where the essential structural elements for the target site of the autogenous regulator protein S8 are almost all within the L5 protein coding region (49).

Amino acid sequence comparison of MvaL1 with L1 equivalent r-proteins from other organisms

Based on the amino acid sequence the M_r of MvaL1 was calculated to be 24.176 kD; with 25 acidic residues (glu, asp) and 37 basic residues (his, lys, arg) this protein is basic.

Amino acid sequence comparison revealed that MvaL1 shares 30.5% (11,79 SD) identity with ribosomal protein L1 from *E.coli* and 41.5% (26,65 SD) and 33.3% (25,62 SD) identity with the L1 equivalent r-proteins from the archaebacteria *H.cutirubrum* and *S.solfataricus* respectively. No eukaryotic counterpart to the L1 equivalent r-protein could be identified so far.

A multiple sequence alignment was carried out for all proteins of the L1 family reported so far. This alignment is unambiguous for the whole protein chain with a few insertions and deletions and gave significant sequence similarity especially for position 135−148 and position 226−228 of the MvaL1 protein with the corresponding regions of the other L1 equivalent r-proteins (Fig.8). These amino acid sequences are almost completely conserved and it is tempting to assume that these two domains are involved in the binding to the 23S rRNA and to its own mRNA. Interestingly, MvaL1 is smaller than *E.coli* r-protein L1, which is the exception to the general finding, that the archaebacterial r-proteins are larger than the equivalent *E.coli* r-proteins (9).

Our results confirm that the organization of r-protein genes from the methanogenic archaebacterium *M.vannielii* clearly follows the operon structure of the *E.coli* genome and is different from the eukaryotic gene arrangement.

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