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A *Mycoplasma* protein homologous to mammalian SRP54 recognizes a highly conserved domain of SRP RNA

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ABSTRACT

A protein homologous to SRP54, a subunit of the mammalian signal recognition particle (SRP), was identified in *Mycoplasma mycoides*. The mycoplasma protein was expressed in *E.coli* and purified to near homogeneity. It was shown to bind specifically *in vitro* to a small mycoplasma RNA with structural features related to the RNA component of SRP. These findings provide evidence of a ribonucleoprotein complex in mycoplasma reminiscent of SRP. A part of the RNA was protected from ribonuclease digestion in the presence of the SRP54 homologue. The protected region contains structural elements that have been highly conserved in SRP RNAs during evolution.

INTRODUCTION

The mycoplasmas are free-living microorganisms with unusually small genomes (600–1500 kb) (1,2). The limited amount of genetic information is reflected in the biochemical repertoire of the organisms. For instance, the mycoplasmas lack many routes of intermediary metabolism present in bacteria with larger genomes. We have previously shown that the population of tRNAs in *M.mycoides* is severely restricted and employs a mode of codon reading which resembles that of mitochondria (3–6). During evolution the mycoplasmas have been under a pressure to eliminate genes and they seem to have retained only the biochemical functions that are essential for survival. Therefore, they may be regarded as 'minimal' living organisms and are valuable experimental tools for the analysis of the biochemical machinery required for an autonomous living system.

We have now extended our investigations of the translational machinery in mycoplasma by examining macromolecules that are related to the mammalian signal recognition particle (SRP). The SRP plays an essential role in the targeting of protein to the membrane of the endoplasmic reticulum (ER) in mammalian cells (for a review see ref 7). SRP recognizes the aminoterminal signal sequence as it emerges from the ribosome and the resulting complex is transferred to a receptor protein located in the membrane of the ER. The SRP from mammalian cells is composed of an RNA molecule (~ 300 nt) and six different polypeptides. One of the polypeptides, SRP54, seems to bind the signal sequence (8,9) as well as the SRP RNA (9,10). The SRP54 is apparently composed of two domains. The aminoterminal G-domain has the sequence elements of a GTP-binding site and the C-terminal M-domain binds the signal sequence as well as the SRP RNA (8–10). The M-domain is unusually rich in methionines and a structural model has been presented where these amino acids are part of a structure which binds to the signal sequence with hydrophobic interactions (11).

It has recently been shown that organisms other than mammals possess macromolecules related to SRP. RNA molecules with a structural motif found also in mammalian SRP RNA have been identified in lower eukaryotes, prokaryotes as well as archaebacteria (Fig. 6) (12,13). The 4.5S RNA of *E.coli* is one example (14). In addition, proteins with amino acid sequences related to those of mammalian SRP54 proteins have been identified in yeast (15,16) and *E.coli* (11,17,18). Particularly noteworthy is the recent demonstration that the *E.coli* protein, *ffh*, forms an SRP-like complex with 4.5S RNA (19,20). Very little is known about the function of this bacterial complex. Evidence has been presented to indicate that it has a function related to that of the mammalian SRP (19,20) but it has also been suggested that the 4.5S RNA has a basic role in the mechanism of protein synthesis (21,22).

We have previously reported the primary structure of a small RNA species from *M.mycoides* that contains the structural domain typical for SRP RNAs (Fig 6) (23). We have attempted to identify molecules that interact with this RNA and thereby elucidate its function. We now report on a mycoplasma protein related to the mammalian SRP54 protein that specifically binds to the mycoplasma RNA *in vitro*.

MATERIALS AND METHODS

Cloning of the gene encoding protein SRPM54

Recombinant DNA techniques are described in Sambrook et al (24). Oligonucleotides were either purchased from Symbicom, Umeå, Sweden or synthesized on a Beckman DNA synthesizer (DNA SM). PCR was carried out as described (24) using 1 μg mycoplasma DNA /μl, 1 μM oligonucleotides and *Taq* DNA polymerase (Promega). The reaction was allowed to proceed for 30 cycles of replication. For the production of a pUC9 library

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of *Bgl*II fragments of *M.mycoides* DNA the vector was linearized with BamHI and ligated to a mixture of *Bgl*II fragments. The ligation mixture was used to transform XLBlue cells (Stratagene). The resulting colonies were screened by hybridization to a cloned PCR fragment radiolabeled using the Multiprime DNA labelling system from Amersham. Restriction fragments of the insert which contained the SRPM54 gene were cloned in M13 vectors and sequenced (25). For computer analysis programs of the University of Wisconsin Genetics Computer Group were used (26).

Expression of SRPM54 in *E.coli*

In order to express SRPM54 in *E.coli* we made use of the system described in ref (27) with PET vectors (Novagene) in combination with BL21(DE3) pLysS as the host strain. The host contains a chromosomal copy of the T7 RNA polymerase gene under the control of the lac promoter. Synthesis of the enzyme is induced by the addition of IPTG to the medium. The PET vectors feature the promoter and transcription terminator of phage T7 together with an *E.coli* translation initiation signal. In order to clone the genes derived from SRPM54 in pET vectors the following procedure was used. An adaptor oligonucleotide was used to introduce a *Bam*HI restriction site at the *Acc*I site being part of the pUC9 polylinker region downstream of the SRPM54 gene in plasmid pUC9/M54. The resulting plasmid was designated pUC9/M54/BamHI. A construct with a *Nde*I site in nucleotide position 1301 (Fig. 2) was obtained in the following way. The *Nde*I site was first introduced by *in vitro* mutagenesis (28) of a fragment of SRPM54 that contains the N-terminal region of SRPM54. The mutation was verified by sequencing and a fragment encompassing the N-terminal end of SRPM54 up to a *Bsp*1286I site at position 1515 was ligated to a fragment from plasmid pUC9/M54/BamHI reaching from the *Bsp*1286I site at position 1515 to the *Bam*HI site. Finally, the resulting *Nde*I/*Bam*HI fragment was isolated and introduced into the expression vector PET3a to obtain the plasmid pET3a/M54.

Purification of SRPM54

For the growth of cells and purification of SRP54 we used a modification of a previously described procedure (29). *E.coli* BL21(DE3) pLysS was transformed with plasmid pET3a/M54. Cells were grown overnight on LB plates with 50 mg/L of ampicillin and 20 mg/L of chloramphenicol and were then used to inoculate a liquid culture (2L) with the same medium. Cells were grown at 37°C until an optical density of 0.4 was reached. The temperature was then shifted to 30°C and growth was allowed to proceed for 2 hours. The cells were finally harvested and stored at −20°C.

For the purification of protein all operations were carried out at +4°C. Cells were suspended in 50 ml of 50 mM Na^+/Hepes pH 7.6, 2 mM EDTA, 10 mM $NaHSO_3$, 2 mM dithiothreitol, 2 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride (PMSF). After the addition of 5 ml 10% NP-40 the mixture was kept on ice for 10 min and 55 ml of 0.4M NaCl, 20% glycerol was then added. The resulting mixture was homogenized with Ultraturrax and centrifugation was for 20 min at 10,000 rpm using a Beckman JA-10 rotor. The supernatant was diluted with 220 ml of a buffer A containing 20 mM Hepes pH 7.6, 50 mM NaCl, 10% glycerol, 1 mM EDTA, 2 mM DTT, 0.1 mM PMSF, 0.1 mM benzamidine. It was loaded to a column of S-Sepharose Fast Flow (Pharmacia) 4.3×13 cm och the column was washed with 200 ml of buffer A. Elution was carried out with the same buffer using a linear gradient of a total volume of 1 L from 50 mM NaCl to 1 M NaCl. Fractions were analyzed by SDS polyacrylamide gel electrophoresis and SRPM54 was shown to elute at approximately 0.5 M NaCl. Fractions containing SRPM54 were pooled and the protein was concentrated by loading it to another column of S-Sepharose, 1.5×3 cm. Protein bound to the column was eluted with buffer A containing 1.0 M NaCl and fractions containing protein were pooled and dialyzed against a buffer containing 20 mM Tris HCl pH 7.5, 0.5 M NaCl, 50% glycerol, 2 mM DTT, 1 mM EDTA. The purified protein was finally stored at −20°C.

Protein samples were analyzed by SDS-polyacrylamide gel electrophoresis (24). For analysis by Western blotting we used a sample of antibody against the *E.coli ffh* protein that was generously provided by Harris Bernstein, Department of Biochemistry and Biophysics, University of California Medical School, San Francisco (19).

Production of mycoplasma SRP RNA by *in vitro* transcription

The oligonucleotides shown in Fig. 4 were phosphorylated and ligated in order to form a 110 bp fragment. This fragment was purified by gel electrophoresis on SeaPlaque agarose (FMC Bio-Products) and cloned into the EcoRI/HindIII sites of M13mp18. After verifying the sequence the EcoRI/HindIII fragments were cloned in the plasmid vector pUC9. The resulting plasmid was denoted pRNM. Transcription *in vitro* of plasmid DNA digested with *Fok*I was carried out essentially as previously described (5) using 40 mM TrisHCl pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol and 0.5 mM concentration each of ATP, UTP, CTP and GTP. For the synthesis of radioactive RNA adenosine 5′-[α-^{32}P]triphosphate (Amersham) was included in the transcription mixture. Labeling of RNAs by ligating radioactive pCp to the 3′ terminus was carried out as described by England et al (30). For the synthesis of tRNA the plasmid pYGLY6 described in ref (31) was used.

Chromatography on immobilized mycoplasma SRP RNA

The mycoplasma SRP RNA homologue, 0.2 mg, was produced by *in vitro* transcription as described above. It was oxidized with periodate (32) and coupled to agarose adipic acid hydrazide (33) as previously described. The protein sample (SRPM54 purified by S-Sepharose chromatography) was mixed with the resin (1 ml) by stirring overnight. The resin was then washed with a buffer containing 10 mM Tris-HCl pH 7.5, 2 mM DTT, 1 mM EDTA, 10% glycerol, 50 mM NaCl. Stepwise elution was finally carried out with the same buffer using increasing concentrations of NaCl.

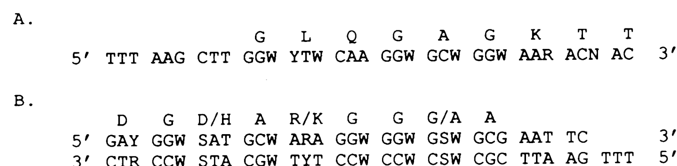

Figure 1. Oligonucleotides used for PCR amplification of part of the gene encoding protein SRPM54. Oligonucleotides are shown together with the corresponding amino acid sequence in SRP54 proteins from eukaryotes and *E.coli* (see Fig 3). The oligonucleotide in A is based on the *E.coli* protein only. Shown in B is the lower DNA strand that was used in PCR as well as the upper complementary strand and its coding sequence. The nucleotide symbols are: W = A or T, S = C or G, Y = C or T, R = A or G. The corresponding sequences in the mycoplasma protein gene are shown in Fig. 2.

```
      D L R A N D L K R S Q E I V E S K S Q R L D A G I L D L E K R K F
   1  AGATCTAAGAGCTAATGATTTAAAAAGAAGTCAAGAAATTGTTGAATCTAAATCTCAACGATTAGATGCTGGAATTTTAGATTTAGAAAAAAGAAAATTT  100

      L V D Q K E E Y L I K L L E D V S G L T K Y Q A K E L L I K Q I K N
 101  TTAGTAGATCAAAAAGAAGAATATTTAATTAAGCTTTTAGAAGATGTGTCAGGTTTAACAAAATATCAGGCAAAAGAATTGTTAATTAAACAAATAAAAA  200

      K S E K E L I S I L K N A E L Q A H S K A K I I S N N I L I L A M
 201  ATAAGTCTGAAAAAGAACTTATCTCTATTTTAAAAAATGCTGAACTACAAGCTCATAGCAAAGCTAAAATTATTTCTAATAATATTTTGATTTTAGCAAT  300

      E R I K V E L T S Q R T T N I V K L P S D D L K G R I I G K D G R
 301  GGAAAGAATTAAAGTAGAACTTACAAGTCAAAGAACAACAAATATTGTTAAATTACCATCAGATGATCTTAAAGGTCGTATTATTGGAAAAGATGGAAGA  400

      N M K T F E Q I G G V D I V V D E T P N V V V V S S F N P I R R E I
 401  AACATGAAAACTTTTGAACAAATCGGTGGAGTTGATATTGTTGTTGATGAAACACCAAATGTTGTTGTAGTTTCTTCATTTAATCCAATTAGAAGAGAAA  500

      A T R T L E Q L I I D G R I Q P V K I E N E L K K Q E Q E L E Y I
 501  TAGCAACACGAACATTAGAACAATTAATTATTGATGGTAGAATTCAACCAGTTAAAATTGAAAATGAGTTAAAAAAACAAGAACAAGAACTAGAATATAT  600

      I Q E T G L N T I K E L N I N D I D I E L V K L I G K L K F R T S
 601  TATTCAAGAAACAGGATTAAATACTATTAAAGAATTAAATATTAATGATATTGATATCGAATTAGTTAAATTAATTGGTAAATTAAAGTTTAGAACTAGT  700

      Y G Q N V L A H S I E V A K L S G A I A S E L G L D V E K A I R A G
 701  TATGGACAAAATGTTTTAGCTCATTCAATTGAAGTTGCTAAATTATCTGGAGCTATTGCTTCTGAGCTAGGTTTAGATGTTGAAAAAGCAATTAGAGCTG  800

      L L H D I G K A I D F E K Q G S H V V L G A E I A R K Y N E D P I
 801  GTTTATTACATGATATTGGTAAAGCAATAGATTTTGAAAAGCAAGGAAGTCATGTAGTATTAGGTGCTGAAATTGCTAGAAAATATAATGAAGATCCAAT  900

      I I N S I E S H H E D K E K S S E I A A I V A I A D S I S A S R P
 901  AATTATCAATTCTATTGAATCACATCATGAAGATAAAGAAAAATCTAGTGAAATAGCTGCTATTGTTGCTATTGCTGATTCTATTTCAGCTTCAAGACCA  1000

      G A R Y N A I D E F I L R M H E I E K I G N S I P G V A K T Y A L Q
1001  GGAGCTAGATATAATGCTATTGATGAATTTATTTTAAGAATGCATGAAATTGAAAAGATTGGAAATTCAATTCCTGGAGTTGCTAAAACTTATGCATTAC  1100

      S G R Q I R L I V D P L V A S D L D L A L I L E K M K E Q I K N K
1101  AATCAGGTCGTCAAATTAGATTAATTGTTGATCCATTAGTTGCTTCTGATTTAGATTTAGCTTTAATTTTAGAAAAAATGAAAGAACAAATTAAAAATAA  1200

      V I I P G E I T I T V I R E K K E T D I L K *
1201  AGTAATTATTCCTGGTGAAATAACTATAACTGTAATAAGAGAAAAAAAAGAAACAGATATTTTAAAATAAATAATTTAATTAGAAATTATTAAAGGAGTT  1300

      M G F G D F L S K R M Q K S I E K N M K N S T L N E E N I K E T L
1301  aatatgGGATTTGGTGATTTTTTATCAAAAAGAATGCAAAAAAGCATTGAAAAAAATATGAAAAATTCTACTTTAAATGAAGAAAACATTAAAGAGACTT  1400

      K E I R L S L L E A D V N I E A A K E I I N N V K Q K A L G G Y I
1401  TAAAAGAAATTAGATTATCATTACTAGAAGCTGATGTTAATATTGAAGCTGCTAAAGAAATTATTAATAATGTAAAACAAAAAGCTTTAGGTGGATATAT  1500

      S E G A S A H Q Q M I K I V H E E L V N I L G K E N A P L D I N K
1501  TTCAGAAGGTGCTAGTGCTCATCAACAAATGATTAAAATTGTTCATGAAGAATTAGTAAATATTTTAGGAAAAGAAAATGCACCTTTAGATATAAATAAA  1600

      K P S V V M M V G L Q G S G K T T T A N K L A Y L L N K K N K K K V
1601  AAACCAAGTGTTGTTATGATGGTTGGACTACAAGGAAGTGGTAAGACTACAACAGCTAACAAACTTGCTTATTTATTAAATAAAAAAAATAAGAAAAAAG  1700

      L L V G L D I Y R P G A I E Q L V Q L G Q K T N T Q V F E K G K Q
1701  TTTTATTAGTTGGATTAGATATTTATAGACCAGGAGCTATTGAACAATTAGTTCAACTTGGACAAAAAACAAATACACAAGTATTTGAAAAAGGAAAACA  1800

      D P V K T A E Q A L E Y A K E N N F D V V I L D T A G R L Q V D Q
1801  AGATCCAGTTAAAACAGCTGAACAAGCTTTAGAGTATGCAAAAGAAAATAATTTTGATGTTGTAATTTTAGATACAGCAGGAAGACTACAAGTTGATCAA  1900

      V L M K E L D N L K K K T S P N E I L L V V D G M S G Q E I I N V T
1901  GTTTTAATGAAAGAACTAGATAATTTAAAGAAAAAAACATCACCAAATGAAATTTTATTAGTTGTTGATGGAATGAGTGGTCAAGAAATTATTAATGTTA  2000

      N E F N D K L K L S G V V V T K L D G D A R G G A T L S I S Y L T
2001  CAAATGAGTTTAATGATAAGTTAAAACTATCTGGAGTTGTTGTTACTAAATTAGATGGAGATGCTAGAGGGGGAGCTACTTTATCTATTAGTTATTTAAC  2100

      K L P I K F I G E G E G Y N A L A A F Y P K R M A D R L M G M G D
2101  TAAATTACCTATTAAGTTTATTGGTGAAGGTGAAGGATATAATGCTTTAGCTGCTTTTTATCCAAAAAGAATGGCCGATAGATTGATGGGAATGGGTGAT  2200

      I E T L F E R A V E N I D E R S I Q K T M N R M F L G Q F D L E D L
2201  ATTGAAACTTTATTTGAAAGAGCTGTAGAAAATATTGATGAACGTTCTATTCAAAAAACTATGAACAGAATGTTTTTAGGTCAATTTGATTTAGAAGATT  2300

      R N Q L A Q I A K M G S L N K L M K M L P I N K V S E S Q I Q E A
2301  TAAGAAACCAATTAGCTCAAATTGCAAAAATGGGTAGTTTAAATAAATTAATGAAAATGTTACCTATTAATAAAGTAAGTGAATCACAAATTCAAGAAGC  2400

      Q R K L A V F S I L M D S M T L K E R R D P R V L K A I S R K N R
2401  TCAAAGAAAATTAGCTGTCTTTTCAATTTTAATGGATTCAATGACTTTAAAAGAAAGACGTGATCCAAGAGTTTTAAAAGCAATAAGTAGAAAAAATCGA  2500

      I I K G S G R S E K E F N E L I N S F E K G K K Q V L E I T K M I K
2501  ATTATTAAAGGATCAGGTCGAAGCGAAAAAGAATTTAATGAATTAATTAATTCATTTGAAAAAGGTAAAAAACAAGTTTTAGAAATCACTAAAATGATTA  2600

      S G R M P N L S K G G F K F * F M K I K I I C F G K L D K K F Y I
2601  AAAGTGGAAGAATGCCAAATCTATCAAAAGGTGGATTTAAATTTTAATTTATGAAAATTAAAATCATTTGTTTTGGTAAATTAGATAAAAAATTTTATAT  2700

      D A F N D Y F K R L E K Y A D I E I I E L K E E I N G E L N K I K
2701  TGATGCTTTTAATGATTATTTTAAAAGATTAGAAAAATATGCTGATATAGAAATAATTGAATTAAAAGAAGAAATAAATGGTGAATTAAATAAAATAAAA  2800

      E L N S D
2801  GAACTAAATTCAGATCT  2817
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Figure 2. Nucleotide sequence of a fragment of *M.mycoides* DNA featuring the gene encoding protein SRPM54. The amino acid sequence corresponding to SRPM54 is shown in bold letters. Also indicated are open reading frames flanking the SRPM54 coding sequence. The sequence altered to a *Nde*I site used for cloning in pET vectors is shown in lower case (nucleotide positions 1301–1306). The sequences in the SRPM54 gene that correspond to the oligonucleotides used for PCR (Fig. 1) are shown underlined.

RESULTS

Cloning of the gene encoding protein SRPM54

An alignment of SRP54 proteins from mammals, yeast and *E.coli* reveals a few regions with significant homology (15). Using these conserved sequence elements of the SRP54 protein family it was possible to construct degenerate oligonucleotides (Fig. 1 and 2) for PCR cloning of the mycoplasma homologue. The degeneracy of the oligonucleotides was kept to a minimum by taking advantage of the unusual codon usage in mycoplasmas (34) and avoiding G and C in the third position of many codons. The oligonucleotides contained HindIII and EcoRI recognition sites to facilitate the cloning of the PCR products into vectors with a polycloning site. A PCR reaction using these oligonucleotides and *M.mycoides* chromosomal DNA resulted in a single amplified fragment, approximately 450 bp in size. This fragment was cloned in M13mp18 and sequence analysis revealed an open reading frame with extensive homology to the *E.coli* SRP54 homologue, *ffh*.

A pUC9 library of *Bgl*II fragments of *M.mycoides* DNA was then screened using the PCR fragment as a probe. Two colonies that hybridized to the probe were selected for further study and were shown to contain the same plasmid with a 2.8 kb *Bgl*II insert. This recombinant was designated pUC9/M54. The nucleotide sequence of the insert is shown in Fig. 2. It contains an open reading frame corresponding to a protein of a predicted molecular weight of 50 kDa. This protein, denoted SRPM54, is homologous to SRP54 proteins previously identified from mammalian cells (11,18), yeast (15), and *E.coli* (17) (Fig 3). It is most closely related to the *E.coli* protein (*ffh*) (41% identical amino acids).

The codon usage in the SRPM54 gene is typical for species of *Mycoplasma* (34). Most notably, there is an abundance of codons with A or U in their third positions (Table I). This is a reflection of the fact that the mycoplasma genome has an A/T-content which is close to 75% (1).

Upstream of the SRPM54 coding sequence is another open reading frame of unknown significance. In between these open reading frames there are no typical bacterial promoter or transcription termination signals. Therefore, it seems likely that the SRPM54 protein gene is part of a transcriptional unit with more than one protein.

```
Can   .MVLADLGRk itSALRs.LS NATIINEEvl naMLKEVCTA LLEADVNIKL VKqLReNVKS aIdlEEMas.
Sp    .MVFADLGRR lnSALgd.FS kATsVNEElV dtLLKnICTA LLEtDVNVrL VqELRsNIKk KInvstLpq.
Sc    .MVLADLGkR inSAvnnaiS NtqddfttsV dvMLKgIvTA LLEsDVNIaL VskLRNNIrS qllsEnrsek
Ec    ..mFdnLtdR lsrtLRnisg rgr.ltEDnV kdtLrEVrmA LLEADValpv VrEFiNrVKe KavghEvnks
Mm    mgfgdfLskR mqksieknMk NsT.lNEEnI ketLKEIrls LLEADVNIea aKEiiNNVKq Kalggyiseg

                                                    I
Can   ...GlNKrKm IQhAVFkELv KLV...dPgV KAWTPtKGKq NVIMfVGLQG SGKTTTCSKL AYYYQRKGWK
Sp    ...GiNgkri VQkAVFdELc sLV...dPkV dAFTPkKGrP sVIMmVGLQG SGKTTTCSKL AlhYQRrGLK
Sc    sTtnaqtkKl IQktVFdELc KLVtcegsEe KAFvPkKrKt NIIMfVGLQG SGKTTsCtKL AvYYskrGFK
Ec    lTpGqefvKi Vrn....... eLVaaMgeEn qtLnlaaqpP aVVLmaGLQG aGKTTsvgKL gkFlreKhkK
Mm    asahqqmiKi Vhe....... eLVniLgkEn apLdi.nkKP sVVMmVGLQG SGKTTTanKL AYLlnkKnkK

                                                              II
Can   tCL.ICADTF RAGAFDQLKQ NAtKARIPFY GSYTEmDPVI IAsEGVEKFK NENFEIIIVD TSGRHkQEDs
Sp    sCL.VaADTF RAGAFDQLKQ NAiKARVPYF GSYTEtDPVV IAkEGVDKFK NDrFDVIIVD TSGRHqQEqe
Sc    vgL.VCADTF RAGAFDQLKQ NAirARIPFY GSYTEtDPak VAeEGInKFK kEkFDIIIVD TSGRHhQEEe
Ec    kvLvVsADvY RpaAikQLet lAeqvgVdFF pSdvgqkPVd Ivnaalkeak lkfYDVllVD TaGRlhvDEa
Mm    kvLlVglDiY RpGAiEQLvQ lgqKtntqvF ekg.kqDPVk tAeqalEyaK enNFDVVIlD TaGRlqvDqv

                                                  III
Can   LFEEMlQVaN AIQPDniVYV MDASIGQAcE aQAKAFKDKv DvasVIVTKL DGHAkGGGAL SAVAATKsPI
Sp    LFaEMveISd AIrPDqtImI LDASIGQAAE sQsKAFKEta DFgaVIITKL DGHAkGGGAL SAVAATKtPI
Sc    LFqEMieISN vIkPnqtImV LDASIGQAAE qQsKAFKEss DFgaIIlTKM DGHArGGGAi SAVAATntPI
Ec    MmDEikQVha sInPvetlFV vDAmtGQdAa ntAKAFnEal pLtgVVlTKv DGdArGGaAL SirhiTgkPI
Mm    LmkELdnlkk ktsPneilLV vDgmsGQeii nvtneFnDKl kLsgVVVTKL DGdArGGatL SisylTKlPI

                         G <--- ---> M
Can   IFIGTGEHID DFEPFktqpF ISKLLGMGDI EGLIDKVnEL .kLDDnEALI EKLKhGQ.FT LRDMyEQFQN
Sp    VFIGTGEHIn DLErFsPrsF ISKLLGLGDl EGLmEhVqsL .DFDkkn.MV knLeqGk.FT vRDFrDQLgN
Sc    IFIGTGEHIh DLEkFsPksF ISKLLGiGDI EsLfEqlqtv snkEDakAtm EniqkGk.FT LlDFkkQMQt
Ec    kFlGvGEktE aLEPFhPdri aSriLGMGDV lsLIEdIesk VDraqaEkLa sKLKkGdgFd LnDFlEQLrq
Mm    kFIGeGEgyn aLaaFyPkrm adrLMGMGDI EtLfEravEn IDersiqktm nrMflGQ.Fd LeDLrnQLaq

Can   IMKMGPFSqi LGMIPGfGTd fMsKgNEQES mARLKKLMtI MDSMnDQELD StDgaKvFsK QPgRIQRVAR
Sp    IMKLGPLSkm asMIPGMs.n mMngmNDeEg slRMKrMLyI vDSMTEQELD S..dgllFVe QPSRVlRVAR
Sc    IMKMGPLSni aqMIPGMs.n mMnqvgEeEt sqkMKKMvyV LDSMTkeELE S..dgrmFIe ePtRmvRVAk
Ec    mknMGgMasl MGklPGMG.q ipdnvksQmd dkvLvrMeaI inSMTmkEra kpE....iIK g.SRkrRIAa
Mm    IaKMGsLnkl MkMlP.in.k vses.qiQEa qrkLavFsil MDSMTlkErr dpr....vlK aiSRknRIik

Can   GSGvStrDVq ELLTQYtKFA QMvK...... ........KM GGiKGLFKGG dMSKNvSqsQ MAKlnQQMAK
Sp    GSGtSVlEVe EtisQvrvFA QMaK...... ........Ki GGKdGiL..G kLggNpaaal kkdprQlaAm
Sc    GSGtSVfEVe miLmQqqmmA rMaqtatqqq pgapganarM pGmpnMpgmp nMpgmpnMPg MpKvtpQMmq
Ec    GcGmqVqDVn rLLkQFddmq rMmKK..... .......... .mKKGgMakm mrSmkgmMPp gfpgr.....
Mm    GSGrSekEfn ELinsFe... ....K..... .......... .GKKqvLeit kMiKsgrMPn LsKggfkf..

Can   mMDPRVLHHM GGMAGLQS.. ........MM rqFQqGAaGn MKGmM.gFNN M......... ..........
Sp    qkrmqaMgmg GGMpGLnpgs mnfgdiskMa nmLmgGggpg gaGgM.dFsg MLnqfQnmQk pprrr.....
Sc    qaqqklkqnp GlMqn..... ........MM nmFggGmgGg MgGgMpdmNe MMkmmQdpQm qqmakqfgmg
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Figure 3. Alignment of *M.mycoides* SRPM54 and related proteins. Shown are sequences from canine (Can) (11,18), *S.pombe* (Sp) (15) *S.cerevisiae* (Sc) (15), *E.coli* (Ec) (17) and *M.mycoides* (Mm). The alignment was generated using the program PILEUP of the University of Wisconsin Genetics Computer Group. Capital letters indicate identical or similar amino acids. G- and M-domains are indicated and in the G-domain the three consensus sequence elements of a GTP-binding domain (I–III) (39) are shown in bold.

Expression of SRPM54 in *E.coli*

To allow a biochemical analysis of the SRPM54 protein we cloned and expressed it in *E.coli*. A system was used based on T7 RNA polymerase described in ref (27) with the vector pET3a in combination with *E.coli* BL21(DE3) as the host organism. The gene for SRPM54 was cloned in pET3a, giving rise to the plasmid denoted pET3a/M54. *E.coli* strain BL21(DE3) pLysS was then transformed with this construct. Expression of the mycoplasma protein severely retarded the growth of the host strain. Nevertheless, analysis by SDS-PAGE showed that 2−3 hours after induction of T7 RNA polymerase a product of the expected size (50 kDa) had been synthesized. It constituted approximately 3% of the total soluble cellular protein. Therefore, the yield of protein was reasonably high in spite of the fact that the codon usage of mycoplasma with a strong bias towards A/U-rich codons differs considerably from that of *E.coli*.

The products of the expression system were analyzed by Western blotting using antibody against the *E.coli ffh* protein (data not shown). This antibody crossreacted with the recombinant mycoplasma protein produced in our expression system. The mobility of this protein was identical to that of a protein in a mycoplasma extract responding to the antibody. These findings suggest that the recombinant protein is identical to an authentic mycoplasma protein.

The SRPM54 protein was purified from a cellular extract using chromatography on S-Sepharose (see Materials and Methods and Fig 7). The purification in this step was approximately 20-fold and analysis by SDS polyacrylamide gel electrophoresis showed that SRPM54 constituted 50−60% of the total protein. The yield of SRPM54 protein was approximately 1 mg from 1 L of growth medium.

Table I. Codon usage of the *M.mycoides* SRPM54 gene

Phe	UUU	15	Ser	UCU	4	Tyr	UAU	7	Cys	UGU	0
Phe	UUC	0	Ser	UCC	0	Tyr	UAC	0	Cys	UGC	0
Leu	UUA	41	Ser	UCA	10	Stop	UAA	0	Trp	UGA	0
Leu	UUG	1	Ser	UCG	0	Stop	UAG	0	Trp	UGG	0
Leu	CUU	2	Pro	CCU	3	His	CAU	2	Arg	CGU	2
Leu	CUC	0	Pro	CCC	0	His	CAC	0	Arg	CGC	0
Leu	CUA	6	Pro	CCA	7	Gln	CAA	22	Arg	CGA	2
Leu	CUG	0	Pro	CCG	0	Gln	CAG	0	Arg	CGG	0
Ile	AUU	30	Thr	ACU	10	Asn	AAU	27	Ser	AGU	9
Ile	AUC	1	Thr	ACC	0	Asn	AAC	4	Ser	AGC	2
Ile	AUA	2	Thr	ACA	8	Lys	AAA	48	Arg	AGA	15
Met	AUG	20	Thr	ACG	0	Lys	AAG	5	Arg	AGG	0
Val	GUU	20	Ala	GCU	20	Asp	GAU	20	Gly	GGU	13
Val	GUC	1	Ala	GCC	1	Asp	GAC	0	Gly	GGC	0
Val	GUA	6	Ala	GCA	5	Glu	GAA	33	Gly	GGA	19
Val	GUG	0	Ala	GCG	0	Glu	GAG	3	Gly	GGG	1

SRPM54 specifically binds mycoplasma SRP RNA

We have previously reported the sequence of a mycoplasma RNA which has a structural motif also found in SRP RNAs (23). This RNA will be referred to as 'SRP RNA' although we do not know whether it is involved in signal recognition. The interaction between the mycoplasma SRP RNA and SRPM54 was studied using RNA produced by *in vitro* transcription. We constructed a synthetic gene where the RNA coding sequence is located immediately downstream of the T7 RNA polymerase promoter (Fig. 4). At the 3′ end of the coding sequence there is a *Fok*I restriction site. This fragment was introduced into pUC9 to produce the recombinant pRNM. The RNA was produced by cleavage of pRNM DNA with *Fok*I followed by transcription with T7 RNA polymerase. The RNA product had the expected size as verified by polyacrylamide gel electrophoresis of radioactively labeled RNA.

The interaction of SRPM54 with SRP RNA was first examined with ribonuclease digestion assays. In these studies we used SRPM54 purified by S-Sepharose chromatography as described above. In one experiment uniformly labeled SRP RNA was incubated with SRPM54 protein and digested with RNase A. A fragment of approximately 45 nucleotides was protected from cleavage (Fig 5A, lane 5). S-Sepharose fractions lacking the 50 kDa SRPM54 protein were not able to protect the RNA from cleavage, indicating that SRPM54 was responsible for the protection against RNase. Furthermore, in a control experiment we made an extract of *E.coli* BL21(DE3) cells transformed with plasmid pET3a without insert. When this extract was fractionated using S-Sepharose chromatography we failed to observe any protection against cleavage by RNase A. The nuclease protection was also specific with respect to the RNA as shown by the following circumstances. A large excess, approximately 10^5-fold, of unlabeled polyU was used in all experiments. Furthermore, when labeled transfer RNA was used instead of SRP RNA we did not observe any protection dependent on SRPM54 (Fig 5A, lanes 3−4 and 7−8).

In order to define the region of the SRP RNA that interacts with the SRPM54 protein we carried out RNase protection experiments using RNA radiolabeled at its 3′ terminus. The results obtained by digestion of the RNA in the absence of protein is shown in Fig 5B, lanes 1−3. The cleavage pattern revealed pyrimidines exposed to the nuclease (also shown in Fig 6). It may be noted that since RNase A has a preference for single stranded regions the observed digestion pattern is compatible with the postulated secondary structure in Fig 6. When nuclease cleavage was carried out in the presence of SRPM54 protein an entirely different pattern was obtained. A region spanning nucleotide positions 18 to 62 was now protected (Fig 5B, lanes 3−6 and Fig 6). The size of the protected region (45 nucleotides) agreed with the size of the uniformly labeled fragment observed in Fig 5A, lane 5. Similar experiments were also carried out with RNase T1 (data not shown). Although this enzyme cleaved poorly at the 5′ end of the unprotected region of the RNA (presumably because this region lacks G residues that are exposed to the enzyme) we observed a comparatively efficient cleavage at G-61

*Hind*III T7 5′ 3′ *Fok*I *Eco*RI
AGCT**TAATACGACTCACTATAG** CCGCGATAAGAATAACATCTG AACGAGTTAGGACCGGAAGGTAGCAGCT ATAAGGAAAAGTGTTCTGTAT TGCGGTAAGATGTAA**CATCC**G
ATTATGCTGAGTGATATCGGCG CTATTCTTATTGTAGACTTGC TCAATCCTGGCCTTCCATCGTCGATATT CCTTTTCACAAGACATAACGC CATTCTACATT**GTAGG**CTTAA

Figure 4. Synthetic gene encoding the mycoplasma SRP RNA. The oligonucleotides used for construction of a synthetic gene for mycoplasma SRP RNA are shown. The T7 RNA polymerase promoter as well as the *Fok*I site are indicated by bold letters.

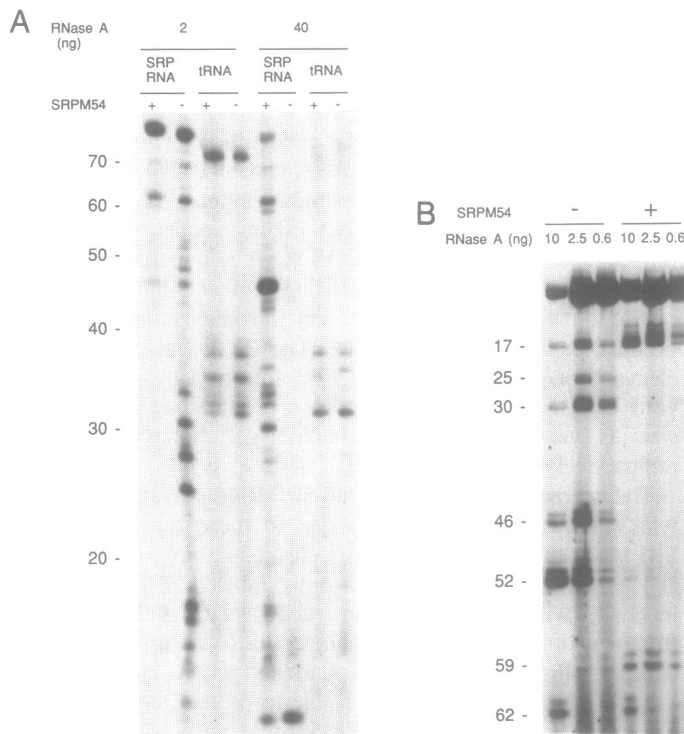

Figure 5. SRPM54 protein protects mycoplasma SRP RNA from ribonuclease cleavage. Radiolabeled RNA (SRP RNA or tRNA as indicated) at a concentration of 10 nM was incubated with or without 0.1 μg of SRPM54 in a mixture containing 1 μg/μl of polyuridylic acid, 40 mM Tris-HCl pH 7.5, 4 mM $MgCl_2$, 10% glycerol, 200 mM NaCl in a final volume of 7 μl. After 1 min at room temperature 1 μl of H_2O was added that contained RNase A in the amounts indicated. After incubation for 15 min at room temperature the mixture was extracted with phenol/chloroform. Carrier tRNA, 2 μg, was then added and the RNA precipitated with ethanol. After centrifugation the pellet was washed with 70% ethanol and finally dissolved in 2 μl 50% formamide and loaded on a 10 % denaturing polyacrylamide gel. *Panel A.* The RNA used was produced by *in vitro* transcription of a synthetic gene using adenosine 5'-[α-^{32}P]triphosphate. Shown to the left of the gel are the positions of size markers. *Panel B.* Experiments were carried out as described above except that the RNA was produced by ligating radiolabeled pCp to to the 3' end of unlabeled RNA (30). The concentration of labeled RNA in the incubation mixture was 80 nM. Bands appear in duplicate since the RNAs were obtained by *in vitro* transcription using T7 RNA polymerase, a procedure which gives rise to products not entirely homogenous at their 3' ends (5). The cleavage observed between nucleotides 59 and 60 in lanes 4−6 is a product of a nuclease present in the preparation of SRPM54 as shown by experiments carried out in the absence of RNase (data not shown). Indicated to the left of the gel are nucleotide positions in the RNA as shown in Fig 6.

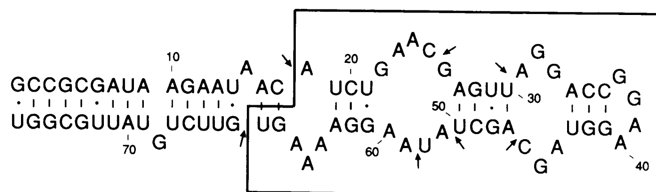

Figure 6. *M.mycoides* SRP RNA homologue and a region of the molecule interacting with SRPM54. The mycoplasma SRP RNA homologue features the structural domain characteristic of SRP RNAs (12,13). The consensus elements of this domain are the nucleotides shown in bold letters as well as a characteristic secondary structure in the right part of the molecule. The arrows indicate the sites of cleavage with RNase A and the region within the box represents the part of the molecule being protected from cleavage in the presence of SRPM54 (Fig 5B).

in the presence of SRPM54. In addition, a low amount of nuclease activity in our preparation of SRPM54 cleaved the RNA on the 3' side of nucleotides 15 and 59 (Fig 5B, lanes 4−6). Taken together our results from the nuclease protection experiments therefore show that the region of the RNA corresponding to nucleotide positions 18−59 is resistant to nuclease and presumably is in more or less close contact with the protein. This part of the SRP RNA contains structural elements that have been well conserved during evolution (12,13). The remaining part of the RNA (to the left in Fig 6), on the other hand, does not seem to interact with the protein. A comparison of bacterial RNAs homologous to mammalian SRP RNA reveals that this part of the RNA has been poorly conserved during evolution and shows no consensus sequence elements (13).

A specific interaction between SRP RNA and SRPM54 was also shown by gel retardation experiments and an assay based on the binding of RNA-protein complexes to nitrocellulose filters (data not shown). In addition, the SRPM54 protein was specifically retarded on a column of immobilized mycoplasma SRP RNA (Fig 7). This column was prepared by covalently attaching the 3' end of the mycoplasma RNA to an agarose matrix (see Materials and Methods). SRPM54 purified by S-Sepharose chromatography was applied to such a column and a majority of the polypeptides in the loaded fraction were eluted with buffer containing 0.3M NaCl (Fig 7, lanes 3−4). On the other hand, SRPM54 remained on the column also in the presence of buffer containing 1.5 M NaCl. When the column finally was eluted with 0.1% SDS only SRPM54 was released (Fig 7, lanes 5−6). These results indicate that SRPM54 interacted very strongly with the SRP RNA.

DISCUSSION

We have made use of conserved sequence elements of SRP54 proteins from mammals, yeast and *E.coli* to clone a related protein from *M.mycoides*. The predicted size of this protein (50 kDa) agrees with the observed molecular weight of a protein that we identified in a mycoplasma extract using antibody against the *E.coli ffh* protein.

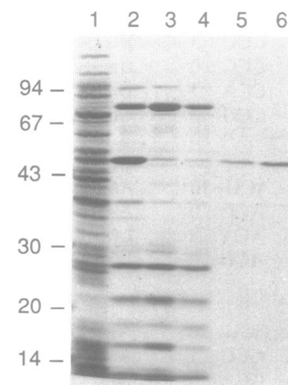

Figure 7. SRPM54 is specifically retarded on a column of immobilized SRP RNA. Samples of protein were analyzed by SDS polyacrylamide gel electrophoresis. An extract of *E.coli* cells producing SRPM54 was prepared (lane 1) and SRPM54 was purified from this extract with S-Sepharose chromatography (lane 2). This material was then loaded on a column of immobilized mycoplasma SRP RNA. Lanes 3−4 show material that was eluted with buffer containg 0.3M NaCl and lanes 5−6 show the protein eluted with 0.1% SDS after the column had been extensively washed with 1.5 M NaCl.

The SRP54 protein from mammalian cells is believed to be composed of two domains (11,18). The N-terminal domain features sequence elements consistent with a GTP binding site. Also in the mycoplasma protein we have found these structural elements and the entire G-domain is extensively homologous to the SRP54 proteins previously identified (Fig. 3). For instance, a comparison to the *E.coli* protein shows 41% identical amino acids. The C-terminal M-domain of mammalian SRP54 is unusually rich in methionines and a model has been presented where these amino acids are part of a structure which recognizes the signal sequence (11). The M-domain appears to be less conserved than the G-domain. A comparison between the M-domains of the *E.coli* and *M.mycoides* proteins shows only 33% identical amino acids. Furthermore, the mycoplasma protein does not contain as many methionines as the SRP54 proteins from eukaryotes and *E.coli*.

In order to examine the biochemical properties of SRPM54 we developed a system for efficient expression and purification of the protein. The codon usage of the mycoplasma SRP54 gene differs considerably from that of *E.coli* (Table I). For instance, in *E.coli* the codon AGA is an unusual codon that is read by tRNAs present at a low concentration. This codon is frequent in the SRPM54 message but there is nevertheless a substantial production of the recombinant protein in our expression system. These results demonstrate that the production of large quantities of mycoplasma proteins in *E.coli* is feasible. In our case we have benefited from the complete absence of the codon UGA which is used as a tryptophan codon in *Mycoplasma* but signifies a stop in the universal code (35).

A variety of techniques were used to examine the specificity in the interaction between the SRPM54 protein and the mycoplasma SRP RNA. Most notably, ribonuclease protection studies as well as an affinity chromatography on a column of immobilized SRP RNA demonstrated a stable and specific binding. These findings strongly suggest that also *in vivo* the protein occurs in a complex with the RNA.

We were also able to define a region of the mycoplasma SRP RNA that interacts with SRPM54. This region corresponds exactly to a domain extremely well conserved in SRP RNAs during evolution. The consensus features of the domain include a typical secondary structure as well as certain conserved nucleotides as shown in Fig 6. One would expect that the 'consensus domain' of the RNA and its binding to the SRP54 protein is functionally important. Consistent with this idea is the observation that SRP RNA homologues from a wide variety of bacteria are able to compensate for a loss of the *E.coli* gene for 4.5S RNA although the only structural similarity between these RNAs is in the 'consensus domain' (36,37).

Evidence has previously been presented that the RNA-binding capacity of the mammalian SRP54 protein resides solely in the C-terminal M-domain (9,10). In accordance with these findings preliminary data in our laboratory obtained with a corresponding fragment of the mycoplasma protein suggests that it efficiently binds the mycoplasma SRP RNA. Furthermore, it appears to protect exactly the same region of the RNA as the full length protein (unpublished observations).

CONCLUDING REMARKS

We have identified a protein in *M.mycoides* that interacts *in vitro* with a mycoplasma RNA structurally related to SRP RNA. These findings suggest that the organism possesses an SRP-like complex of a type previously identified in *E.coli* (19,20). Mycoplasmas are extremely simple prokaryotes that presumably have retained a minimal number of genes that are all required for survival. This would suggest that such SRP-like complexes are ubiquitous among bacterial species and that they serve an essential function. Consistent with this notion is the fact that the 4.5S RNA is required for viability of *E.coli* (38) and that related RNAs have been found in a wide variety of bacterial species (13).

In order to examine the function of the mycoplasma ribonucleoprotein complex it will be necessary to identify additional molecules that interact with this complex. If it has a function in secretion or targeting proteins to membranes the mycoplasmas will become attractive experimental organisms for the functional analysis of the SRP complex since they have a simple membrane system as well as a restricted biochemical machinery in general.

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