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# An archaeal protein homologous to mammalian SRP54 and bacterial Ffh recognizes a highly conserved region of SRP RNA

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**Abstract The gene encoding the 54 kDa protein of signal recognition particle (SRP54) in the hyperthermophilic archaeon *Pyrococcus furiosus* has been cloned and sequenced. Recombinant *P. furiosus* SRP54 (pf-SRP54) and the N-terminal G-domain and C-terminal M-domain (pf-SRP54M) of pf-SRP54 with an amino-terminal addition of six histidine residues were expressed in *Escherichia coli* and subjected to binding experiments for SRP RNA, non-conserved 213-nucleotide RNA (helices 1, 2, 3, 4 and 5) and conserved 107-nucleotide RNA (helices 6 and 8) from SRP RNA. The RNA binding properties of the purified protein were determined by filter binding assays. The histidine-tagged pf-SRP54M bound specifically to the conserved 107-nucleotide RNA in the absence of pf-SRP19, unlike the eukaryotic homologue, with an apparent binding constant (*K*) of 18 nM. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.**

*Key words:* Signal recognition particle; 7S RNA; 54 kDa protein of signal recognition particle; RNA binding protein; *Pyrococcus furiosus*

## 1. Introduction

A study of the phylogenic tree based on ribosomal RNA or protein sequences shows that all known organisms are related, suggesting a common ancestor. All organisms can be divided into three groups: eukarya, bacteria, and archaea [1]. According to structural studies on ribosomal proteins, protein elongation factors, protein initiation factors and RNA polymerase, the archaea form a specific group that are apparently different in evolution, and most of the proteins of this group as gene products are much more analogous to those of eukarya [2–10].

A signal recognition particle (SRP) is a ribonucleoprotein complex involved in the translocating of secretory proteins to cellular membranes by recognizing signal sequences as they appear on the surface of translating ribosomes [11]. SRP-directed protein targeting was elucidated first in eukarya where the particle consisted of one SRP RNA molecule (~300 nucleotides) and six proteins (SRP9, SRP14, SRP19, SRP54, SRP68 and SRP72) [12,13]. The prominent role of SRP19 in the assembly of the eukaryotic SRP is supported by its intimate association with SRP RNA [14] and by the finding that SRP54 binds only to the SRP19–RNA complex and not to isolated SRP19 on SRP RNA [15].

Only two molecules, 4.5S RNA and protein Ffh, a homologue of eukaryotic SRP54, have been identified in bacterial SRP [16].

Crystal structures have been determined for the human SRP54 methionine-rich M-domain [17], and the *Escherichia coli* Ffh–4.5S RNA complex [18], thus enhancing our understanding of protein–SRP RNA and protein–signal peptide interactions on the molecular level.

In contrast, in archaea, relatively little is known about the role of SRP in protein targeting and secretion. All three groups of life appear to contain proteins with signal sequences designed according to common particles to indicate shared recognition mechanisms. In agreement with this assertion, archaeal SRP RNA secondary structures are similar to those of eukarya [19]. Furthermore, a SRP-like complex has been found to characterize in the archaea *Archaeoglobus fulgidus* [20] and *Acidianus ambivalens* [21].

Here we describe the cloning, expression, purification and interaction of the hyperthermophilic archaeon *Pyrococcus furiosus* SRP54 (pf-SRP54) and SRP RNA (pf-SRP RNA) from recombinant components.

We established that an archaeal protein (pf-SRP54) homologous to mammalian SRP54 specifically binds to the archaeal pf-SRP RNA in vitro, and that the association is predominantly between the methionine-rich domain of SRP54 (pf-SRP54M) and a highly conserved 107-nucleotide region of SRP RNA (c. pf-SRP RNA) in the absence of SRP19 (pf-SRP19).

## 2. Materials and methods

### 2.1. Bacterial strains, plasmids, and media

*P. furiosus* JCM 8422 was obtained from the Japan Collection of Microorganisms, RIKEN, Japan. Cells were grown at 98°C in a complex medium based on artificial seawater (Jamarine Laboratory, Osaka, Japan) supplemented with 0.5% yeast extract, 1% bacto-tryptone, and 1% soluble starch per liter under strictly anaerobic conditions as described previously [22].

Cloning, expression, and transcription of *P. furiosus* DNA were carried out by using the *E. coli* host/vector systems of JM109/pUC18 or pUC19, BL21(DE3)pLysS/pET15b (Novagen) and

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*Abbreviations:* SRP, signal recognition particle; SRP54, 54 kDa protein of signal recognition particle; SRP19,19 kDa protein of signal recognition particle; SRP RNA, signal recognition particle RNA; pf-SRP RNA, *Pyrococcus furiosus* signal recognition particle RNA; pf-SRP54, *Pyrococcus furiosus* SRP54; pf-SRP54G, GTP binding domain in pf-SRP54; pf-SRP54M, methionine-rich domain in pf-SRP54; c. pf-SRP RNA, conserved region of pf-SRP RNA; nc. pf-SRP RNA, non-conserved region of pf-SRP RNA

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JM109/pGEM-3Z. *E. coli* strains were grown in yeast/tryptone medium at 37°C with 100 μg/ml ampicillin.

### 2.2. Cloning and nucleotide sequences of genes encoding pf-SRP RNA and pf-SRP54

On the basis of the nucleotide sequences of the genes for the 5′- and 3′-terminal parts of the conserved sequences of SRP RNA from related archaeal species *Pyrococcus horikoshii* [23] and *Thermococcus celer* [24], two oligonucleotide primers R1 (5′-CCGGAATTCGGCGGCGGGCTAGGCCGGGG-3′) and R2 (5′-CCCAAGCTTGGCGGCGGGCACGCCCGAGG-3′) were designed and PCR amplifications were done to complete the nucleotide sequence of the gene encoding pf-SRP RNA.

During the course of isolation work for translation factor genes in *P. furiosus* by using oligonucleotide fragments from the conserved GTP binding amino acid sequence, a homologous gene of both bacterial Ffh and eukaryotic SRP54 was obtained by chance from this organism and sequenced. The DNA sequence of pf-SRP54 was deposited to DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB057373.

### 2.3. Expression and purification of pf-SRP54, pf-SRP54G and pf-SRP54M

To express pf-SRP54, the DNA fragment encoding the entire protein was amplified by PCR using suitable primers, P1 (5′-CTTAAGGCATATGGTTCTAGATAACCTAGGGAAAG-3′) and P2 (5′-TTGGGATCCTTACATACCAAACCTCCTGGCAAG-3′). The 5′-primer carried a *Nde*I site while the 3′-primer had a *Bam*HI restriction site. The PCR-amplified DNA was gel-purified, cleaved with the two enzymes and ligated into pET15b (Novagen) that had been cut with *Nde*I and *Bam*HI, resulting in plasmid pET-pfSRP54. In this plasmid, six histidine residues were attached in the frame to the amino-terminus of the protein.

To express pf-SRP54G, the DNA region corresponding to the G-domain of pf-SRP54 (amino acid residue positions 1–294) was amplified by PCR with oligonucleotides P1 and P3 (5′-TTGGGATCCTTACAAGAGTCTTGAAACAAATCTCGG-3′) as primers and ligated into pET15b, resulting in plasmid pET-pfSRP54G.

To express pf-SRP54M, the DNA region corresponding to the M-domain of pf-SRP54 (amino acid residue positions 295–443) was amplified by PCR with oligonucleotides P4 (5′-CTTAAGGCATATGCTTGGAGATATTCAAGGATTGC-3′) and P2 as primers and ligated into pET15b, resulting in palsmid pET-pfSRP54M.

For expression of recombinant proteins pf-SRP54, pf-SRP54G or pf-SRP54M, three colonies of *E. coli* BL21(DE3)pLysS harboring respectively the constructed plasmids pET-pfSRP54, pET-pfSRP54G or pET-pfSRP54M were cultured on an LB plate in the presence of 100 μg/ml ampicillin. A single colony was inoculated into 3 ml LB medium containing 100 μg/ml ampicillin, then incubated for 16 h at 37°C. A 1.5 ml inoculum of the overnight culture was added to 100 ml of LB medium containing 100 μg/ml ampicillin, then cultured to $OD_{660} = 0.6 \sim 1.0$ at 37°C and induced with 1 mM IPTG for 2–4 h at 37°C. Purification of histidine-tagged proteins was carried out with Ni-NTA agarose beads using MagExtractor kit (Toyobo).

### 2.4. Synthesis of pf-SRP RNA, c. pf-SRP RNA or nc. pf-SRP RNA

A 314-bp DNA fragment, encoding the mature pf- SRP RNA studied in this work, was amplified with the oligonucleotides R1 and R2. These two primers were designed to create *Eco*RI and *Hin*dIII sites at the 5′- and 3′-ends of the resulting PCR product. The purified product was digested with both *Eco*RI and *Hin*dIII, then inserted into the *Eco*RI–*Hin*dIII sites of pGEM-3Z (pGEM-SRP RNA).

A 107-bp DNA fragment, encoding the c. pf-SRP RNA (helices 6 and 8 of residues G129–C235) of the SRP RNA, was amplified with oligonucleotides R3 (5′-CCGGAATTCGGGGTGCCCGGTGGGGGAGGCACGGCT-3′) and R4 (5′-CCCAAGCTTGCGTGCTCCGTGGCCCCTACCGCTGCT-3′).

A 213-bp DNA fragment, encoding the nc. pf-SRP RNA (helices 1–5) of the SRP RNA, was constructed by ligation of two PCR products, residues 1C–128C from the 5′-end and residue 236T–314C from the 3′-end of SRP RNA. The 5′-end DNA fragment was amplified by PCR using the oligonucleotides R1 and R5 (5′-CCGGGATCCGCGGGACGAGGACTC-3′), which contained a *Bam*HI site, then digested with *Eco*RI and *Bam*HI. The 3′-end DNA fragment was also amplified by PCR using the oligonucleotides R2 and R6 (5′-CCGGGATCCTCGCGGGGGTGCGGG-3′), which contained a *Bam*HI site, then digested with *Hin*dIII and *Bam*HI. These two DNA fragments were ligated and inserted into the *Eco*RI–*Hin*dIII sites of pGEM-3Z.

The pGEM-SRP RNA or constructed plasmids for RNA transcription were linearized by digestion with *Hin*dIII. Labeled RNA was produced in the presence of [$^{32}$P]CTP (Amersham; specific activity 800 mCi/mol) using a MAXIscript in vitro transcription kit supplied by Ambicon Co., Ltd. with linearized pGEM-3Z derivatives. The resulting RNA fragment in the reaction was treated with DNase I and purified using a NucTrap Push column (Stratagene) or by 6% denaturing urea polyacrylamide gel electrophoresis (PAGE).

Unlabeled SRP RNA for competition experiments was produced using a MAGAshortscript kit (Ambicon) with linearized pGEM-3Z derivatives and purified by denaturing urea PAGE.

### 2.5. Protein binding assay

For binding assays, the pf-SRP RNA or c. pf-SRP RNA labeled with $^{32}$P ($\sim 10^4$ cpm) was incubated with various concentrations of pf-SRP54 or their derivatives (0.1–300 nM) in 50 μl of binding buffer (50 mM Tris–HCl, pH 7.6, 10 mM $MgCl_2$, 300 mM KCl, 1 μg tRNA, 1 μg bovine serum albumin (BSA), and 5% glycerol) for 15 min at 37°C. The reaction mixtures were then diluted with 0.5 ml of binding buffer. The mixtures were passed through nitrocellulose filters (Advantec, 0.45 μm pore size; Toyo Roshi Kaisha, Tokyo, Japan), which were washed two times with 300 μl binding buffer. The levels of

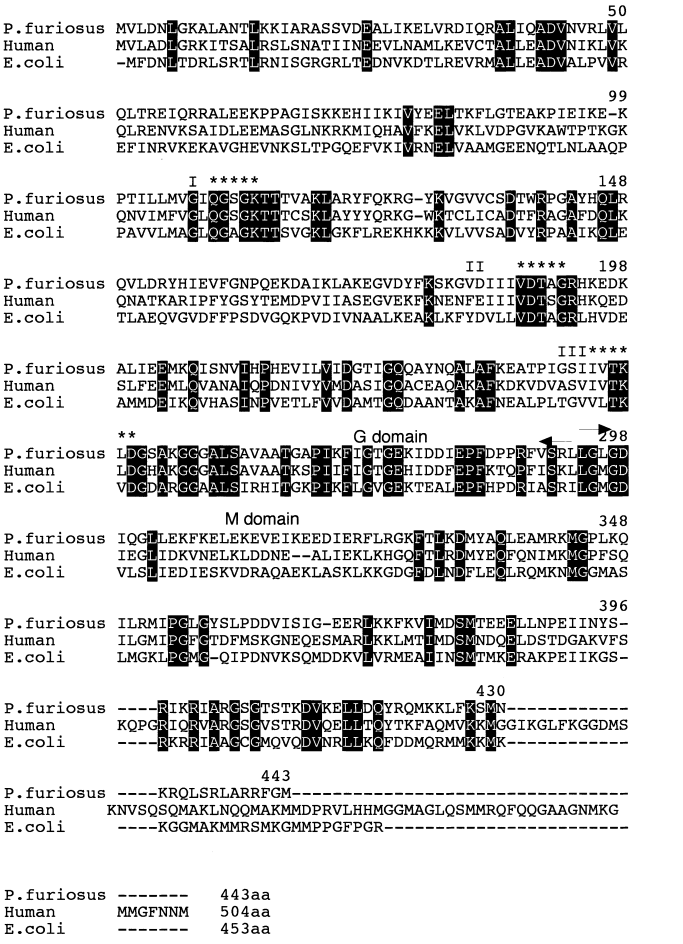


Fig. 1. Alignment of pf-SRP54 and related proteins. Shown are sequences from *P. furiosus*, human [25] and *E. coli* [26]. G- and M-domains are indicated and in the G-domain the three consensus sequence elements of a GTP binding domain (I–III) are indicated by asterisks. Highly conserved residues are shown in reverse print on the black boxes.

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                                                                                                                55
P.furiosus  -----CGGGCUAGGCCGGGGGGGUUCGGCGUCCCCUGUAACCGGAAACCGCCGAUAUGCCG
T.celer     GGCGGCGGGCUAGGCCGGGGGGGUUCGGCGUCCCCCUGUAACCGGAAACCGUCGAUACGCCG
human       --GCCGGGCGCGGUGGCGCGUGCCUGUAGUCCCAGCUACUCGGGAGGCUGAGGCUGAGGA
B.subtilis  ----------------------------------------------UUGCCGUGCUAAG
E.coli      ------------------------------------------------------------
                                                                                                              115
P.furiosus  GGGCCGAAGCCCGGGGGGCGGUUCCCAAAGCCGCUCCCAGAAGCCGAGGUCGAACGAUGA
T.celer     GGGCCGAAGCCCGGGGG-CGGUUCCCGAAGCCGUUCCCGGAAGCCGGGGCACAACGGUGA
human       UCGCUUGAGUCCAGGAGUUCUGGGCUGUAGUGCGCUAUGCCGAUCGGGUGUCCGCACUAA
B.subtilis  CGGGGAGGUAGCGGUGCCCUGUACCUGCAAUCCGCUCUACAGGGCCGAAUCCCUUUCGAG
E.coli      ------------------------------------------------------------
                                                                                                              175
P.furiosus  GUCCUCGUCCCGCGGGGUGCCCGGUGGGGGAGGCACGGCUGAAGGGCCGUGCUAACCCCC
T.celer     UCCCUCGUCCCACGGGGCCGGCGGUGGGCGGGGUCCGGCUGGAGGGCCGGGCUAACGCCC
human       GUUCGGCAUCAAUAUGGUGACCUCCCGGGAGCGGGGGACCA----CCAGGUUGCCUAAGG
B.subtilis  GUUCGUUUACUUUAAGGCCUGCCUUAAGUAAGUGGUG-UUGACGUUUGGGUCCUGCGCAA
E.coli      -----------------------------GGGGGCU-CUG----UUGGUUCUCCCGCAA
                                                                                                              234
P.furiosus  UUUGGGCCC-CGAACCCCGCAAGGCCCGGAAGGGAGCAGCGGUAGGGGCCACGGAGCACG
T.celer     UUUGCCCGC-CGAACCCCGUCAGGCCCGGAAGGGAGCAGCGGUAGGCGGGACGUUCGGCG
human       AGGGG-----UGAACCGGCCCAGGUCGGAAACGGAGCAGG-UCAAAACUCCCGUCUGAUC
B.subtilis  UGGGAAUUCAUGAACCAUGUCAGGUCCGGAAGGAAGCAGCAUUAAGUGAAACCUCUCAUG
E.coli      CGCUACUCUGUUUACCAGGUCAGGUCCGGAAGGAAGCAGC--CAAGGCAGAUGACGCGUG
                                                                                                              291
P.furiosus  C-UCGCGGGGGUGCG---GGCAUGAGAUAGGCCUCGGUGAUGGGAGCGGUGGAGGGUUCC
T.celer     C-UCGUGGGGUAGCG---GGCGUGAGCGAGCCCCGGUGGAAGGGGACGGUGGAGGGUCCC
human       AGUAGUGGGAUCGCGCCUGUCAAUAGCCACUGCACUCCAGCCUGUGCAACAUAGCGAGAC
B.subtilis  UGCCGCAGGGUUGCCU--GGCCCGAGCUAACUGCUUAAGUAACGCUUAGGGUAGCGAAUC
E.coli      UGCCGGGAUGUAGCU----GCCAGGGCCCCCACC--------------------------
                                                                      314
P.furiosus  CACCCUCGGGCGUGCCCGCCGCC       314
T.celer     CACCCCCGGGCGCGCCCGCCGCC       317
human       CCCGUCUCUU-----------         298
B.subtilis  GACA---GAAGGUGCACGGUA--       268
E.coli      ----------------------        113
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Fig. 2. Alignment of SRP RNA sequences. Shown are sequences from *P. furiosus*, *T. celer* [24], human [27], *Bacillus subtilis* [28] and *E. coli* [29]. Highly conserved residues are shown in reverse print on the black boxes.

radioactivity remaining on the nitrocellulose filters were determined by liquid scintillation counting.

## 3. Results and discussion

### 3.1. Cloning, sequencing and identification of the pf-SRP54 gene

During the course of isolation work for translation factor genes in *P. furiosus* by using oligonucleotide fragments predicted from the conserved GTP binding amino acid sequence, a homologous gene to eukaryotic SRP54 and bacterial Ffh was obtained from this organism. This open reading frame is composed of 1329 bp and encodes a protein of 443 amino acid residues (molecular weight = 49.9 kDa).

The pf-SRP54 gene was identified by comparing its predicted protein sequence with sequences of known SRP54 proteins from various organisms (Fig. 1). Pf-SRP54 was most similar to the SRP54 protein of the methanogenic archaeon

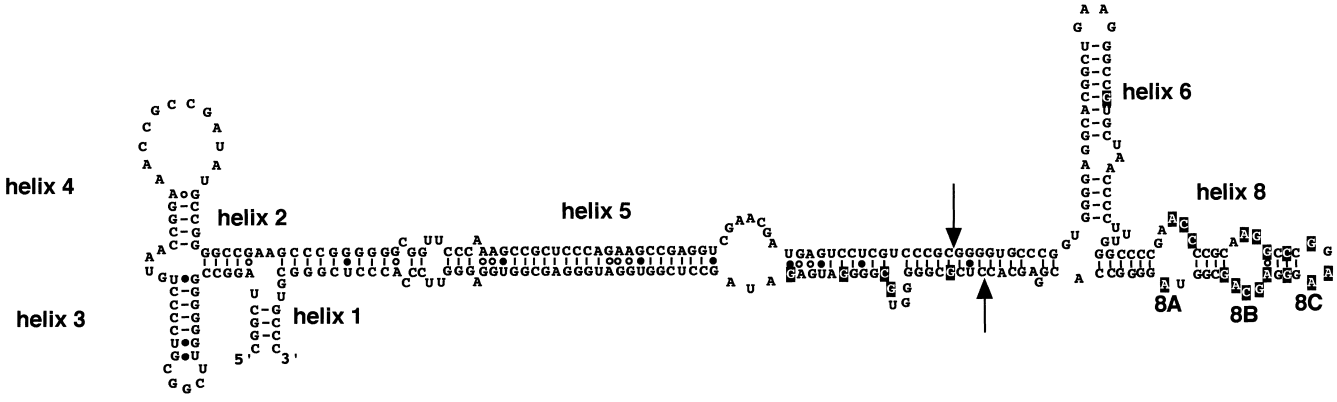


Fig. 3. Secondary structure of pf-SRP RNA. The 5′- and 3′-ends of the RNA molecule are labeled as such; helices are marked 1–8 from the 5′-end according to the nomenclature of Larsen and Zwieb [19]; highly conserved residues are shown in reverse print on the black boxes. Perfect base pairs are connected with a line, and G–U and A–G pairs are shown with a filled and an open circle, respectively. Arrows indicate the borders between the conserved domain of RNA (right) and the rest of the molecule (non-conserved RNA, left). Helix 7 consisting of two base pairs in human is absent from archaeal *P. furiosus*.

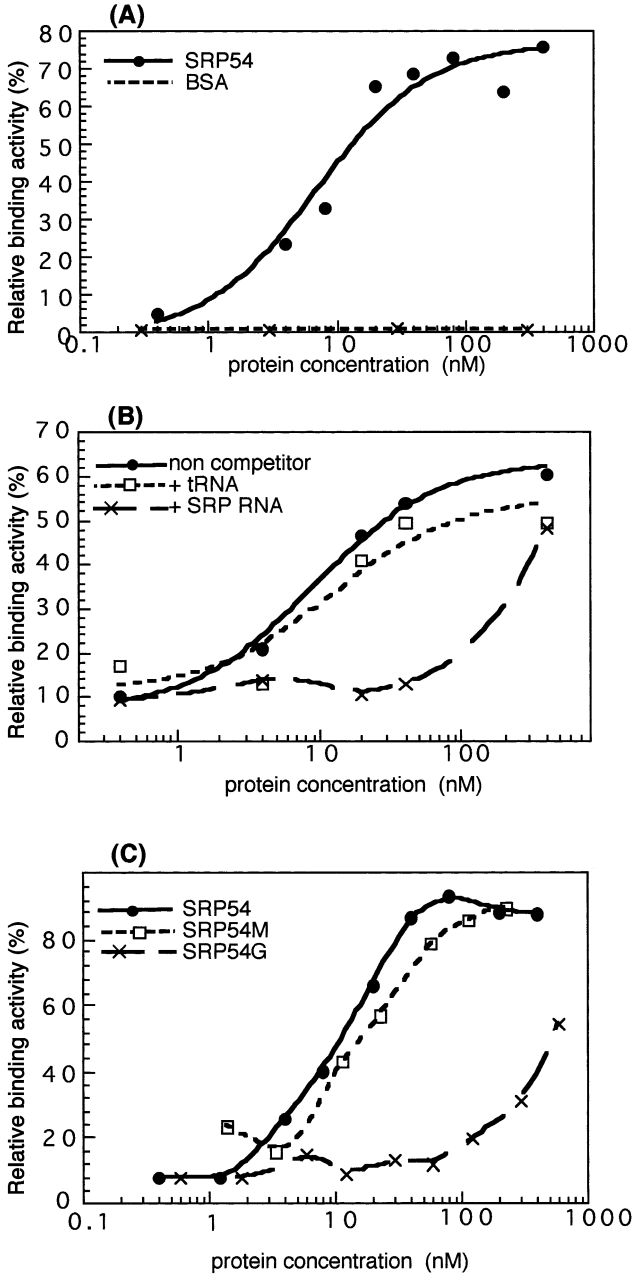


Fig. 4. Nitrocellulose filter binding assay of pf-SRP54, pf-SRP54G and pf-SRP54M. A: Various concentrations of histidine-tagged SRP54 or BSA were incubated with $^{32}$P-labeled SRP RNA, then filtered. After three washes, SRP RNA retention on the filters was monitored. B: The SRP54 and $^{32}$P-labeled SRP RNA complex were formed in the presence of non-labeled tRNA or SRP RNA as a competitor (200 ng). C: Filter binding was carried out with various concentrations of histidine-tagged SRP54G or SRP54M and labeled SRP RNA. Percent of RNA bound (%) was calculated as follows: labeled RNA retained on filter (cpm)×100/total RNA added (cpm). The percent of RNA bound was plotted against the concentration of protein applied in the assay.

*Methanococcus jannaschii*, with 56% sequence identity and 85% sequence similarity. Pf-SRP54 also had 38% identity (72% similarity) to human SRP54 and 30% identity (71% similarity) to *E. coli* Ffh.

The protein is composed of an N-terminal G-domain (1–294 residues) including the conserved GTP binding amino acid sequence motif and a C-terminal methionine-rich M-domain (295–443 residues) (Fig. 1). The three sequence motifs typical for the GTP binding proteins were located in the segments 109QGSGK113, 188VDTAG192 and 245IVTKLD250. The methionine content (nine residues) of pf-SRP54M is also significantly higher than of pf-SRP54G, but contains a lower amount compared to eubacterial Ffh and eukaryal SRP54 (20–25 residues).

### 3.2. Cloning, sequencing and comparative alignment of the pf-SRP RNA

On the basis of the nucleotide sequences of the genes for the 5′- and 3′-terminal parts of the conserved sequences of SRP RNA from related archaeal species *P. horikoshii* [23] and *T. celer* [24], two oligonucleotide primers R1 and R2 (see Section 2) were designed and PCR amplifications were done to complete the nucleotide sequence of the gene encoding SRP RNA. The nucleotide sequence of five types of SRP RNA including *P. furiosus* is shown in Fig. 2. Sets of the same nucleotide among bacteria, archaea and eukaryotes were identified in the middle region of the sequence (positions 188–256 in *P. furiosus* numbering). The derived secondary structure model is presented for *P. furiosus* (Fig. 3). The structure closely corresponds to those proposed for the 7S RNAs of *Halobacterium halobium* [30] and *T. celer* [24]. The secondary structure consists of seven major helical regions, labeled 1–8 as indicated in other SRP RNAs. Helix 8 encompasses the most highly conserved region of the molecule in terms of the primary sequence. The unpaired sequences labeled 8A, 8B and 8C in helix 8 form strongly conserved sequence elements. Studies of bacterial SRP indicate that this portion is required for association with the SRP54 homologue [31]. Site-directed mutagenesis experiments from human SRP RNA showed that SRP19 specifically recognizes the loop motif located in helix 6 and the binding of SRP19 induces the subsequent association of SRP54 to helix 8 [15].

The strong conservation of sequence in this region among eukarya, bacteria, and archaea suggests that within helix 8, a specific primary sequence, as well as secondary structure, is necessary to maintain functional capacity.

### 3.3. Binding of pf-SRP54 or pf-SRP54M to SRP RNA in vitro

In order to examine the binding affinity of SRP RNA for SRP54, the gene was expressed in *E. coli* under the control of a phage T7 promoter. Induction of the *E. coli* strain

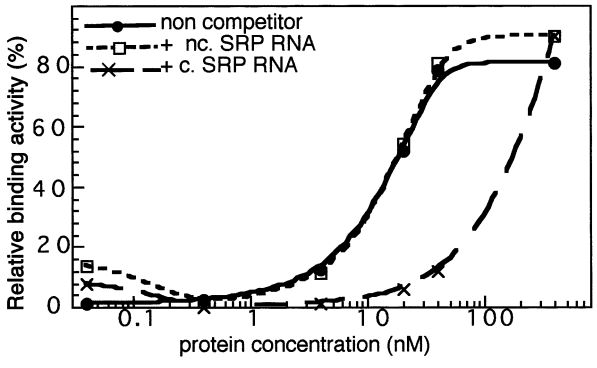


Fig. 5. Nitrocellulose filter binding assay of c. pf-SRP RNA. The SRP54 and $^{32}$P-labeled SRP RNA complexes were formed in the presence of non-labeled nc. pf-SRP RNA or c. pf-SRP RNA as competitors (200 ng).

BL21(DE3)pLysS, transformed with the vector pET-pfSRP54, resulted in the production of a protein with a molecular mass of 54 kDa as seen from *E. coli* crude extracts analyzed on a sodium dodecyl sulfate (SDS) gel stained with Coomassie blue. After purification of the histidine-tagged protein using a Ni-NTA resin column, the 54 kDa band was detected as a single band on SDS gel.

Using the radiolabeled SRP RNA probe transcribed in vitro from a *Hin*dIII-linearized pGEM-SRP RNA, the dose dependence of pf-SRP54 binding to pf-SRP RNA was assayed by a filter binding assay. Under the conditions used, about 80% of the input RNA was retained on the filter at high protein concentrations (Fig. 4A), showing that the SRP54 bound to the SRP RNA with an apparent binding constant of 18 nM. In contrast, BSA manifested no specific binding to the SRP RNA.

When a relatively small amount of unlabeled SRP RNA (Fig. 4B) was incubated with labeled RNA and protein, competition was clearly evident and the complex of labeled RNA was largely dissociated. In contrast, when an equal amount of unrelated tRNA was incubated with labeled RNA and protein, no competition was evident. These results indicated that pf-SRP54 specifically interacts with pf-SRP RNA.

To find the RNA binding region of SRP54 protein, purified defective proteins were used in the RNA binding assay. SRP54M lacks most of the G-domain, and it bound to SRP RNA in the filter assay (Fig. 4C). In contrast, SRP54G lacking the M-domain had no affinity for SRP RNA. Therefore, like mammalian SRP54 and eubacterial Ffh, the RNA binding domain of pf-SRP54 is also located in the M-domain.

### *3.4. Binding of pf-SRP54 to c. pf-SRP RNA in vitro*

In order to define the region of SRP RNA that interacts with SRP54 protein, we carried out a filter binding experiment using c. pf-SRP RNA (positions 129G–235C) of SRP RNA. Under the same conditions used with mature SRP RNA, about 80% of the input c. pf-SRP RNA was retained on the filter at high SRP54 concentrations, showing that SRP54 bound to c. pf-SRP RNA with almost the same binding constant as mature SRP RNA (data not shown). To obtain an estimate of the binding specificity of SRP54 to c. pf-SRP RNA, we compared the ability of unlabeled c. pf-SRP RNA and nc. pf-SRP RNA to compete a fixed amount of $^{32}$P-labeled mature SRP RNA (Fig. 5). As expected, the unlabeled c. pf-SRP RNA competed efficiently, and within the excess of unlabeled nc. pf-SRP RNA an RNA–protein complex was still detectable. These data indicate that pf-SRP54 binds specifically to c. pf-SRP RNA.

Finally, pf-SRP54, unlike its eukaryotic homologue, has significant affinity for 107 conserved RNA nucleotides from pf-SRP RNA in the absence of pf-SRP19. There is overall good agreement between the binding specificity observed for the pf-SRP54/SRP RNA complex presented here and the Ffh/4.5S RNA complex from *E. coli*, although bacterial SRP has a small RNA lacking in helix 6 and no SRP19 homologue unlike archaea [32]. The apparent binding constant ($K$) for formation of the pf-SRP19/SRP RNA complex as determined by filter binding is $\sim$0.5 nM (unpublished data), supporting the possibility of conformational change in the conserved RNA induced by SRP19 in the SRP assembly process as proposed for the eukaryotic system [25].

It has recently been shown that SRP19 from the hyperthermophilic, sulfate-reducing archaeon *A. fulgidus* is capable of binding SRP RNA and promoting incorporation of SRP54 protein into the complex by inducing conformation changes in the proximal asymmetric bulge of helix 8 in SRP RNA [20,32].

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