RNA chaperone activity of L1 ribosomal proteins: phylogenetic conservation and splicing inhibition

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ABSTRACT

RNA chaperone activity is defined as the ability of proteins to either prevent RNA from misfolding or to open up misfolded RNA conformations. One-third of all large ribosomal subunit proteins from *E. coli* display this activity, with L1 exhibiting one of the highest activities. Here, we demonstrate *via* the use of *in vitro trans-* and *cis*-splicing assays that the RNA chaperone activity of L1 is conserved in all three domains of life. However, thermophilic archaeal L1 proteins do not display RNA chaperone activity under the experimental conditions tested here. Furthermore, L1 does not exhibit RNA chaperone activity when in complexes with its cognate rRNA or mRNA substrates. The evolutionary conservation of the RNA chaperone activity among L1 proteins suggests a functional requirement during ribosome assembly, at least in bacteria, mesophilic archaea and eukarya. Surprisingly, rather than facilitating catalysis, the thermophilic archaeal L1 protein from *Methanococcus jannaschii* (MjaL1) completely inhibits splicing of the group I *thymidylate synthase* intron from phage T4. Mutational analysis of MjaL1 excludes the possibility that the inhibitory effect is due to stronger RNA binding. To our knowledge, MjaL1 is the first example of a protein that inhibits group I intron splicing.

INTRODUCTION

The bacterial ribosome is a three millionDa macromolecule, which consists of three RNA molecules—with the 23S rRNA consisting of 2900 nt in *Escherichia coli*—and more than 50 proteins. Large RNA molecules such as the 23S rRNA have a tendency to misfold *in vitro* and become trapped in non-native structures due to a multitude of base-pairing possibilities. Interestingly, the ribosome can be assembled *in vitro* from its individual components—the RNA and the ribosomal proteins—in the absence of ancillary factors without becoming trapped in non-native conformations, suggesting that all the information for correct assembly is contained within the ribosomal RNA and proteins. Therefore, it was not surprising when it was shown that one-third of all large ribosomal subunit proteins from *E. coli* have strong RNA chaperone activity (1). Chaperone activity is generally defined as either preventing RNA from misfolding or resolving misfolded RNA structures (2,3). It was suggested that this observed RNA chaperone activity of the ribosomal proteins could play a role during assembly and/or during translation. In addition, it could also be possible that the observed RNA chaperone activity plays an extra-ribosomal role as was suggested previously for various ribosomal proteins (4). However, if the RNA chaperone activity of ribosomal proteins does indeed play an important role in either ribosome assembly, translation or in extra-ribosomal functions, then it is likely to be conserved among different species.

Ribosomal protein L1, designated L10a in eukarya, is a ~25 kDa protein of the large ribosomal subunit, which is highly conserved among all three domains of life. It localizes to the stalk region proximal to the E-site where the deacylated tRNA is ejected. In archaea and bacteria, L1 has a dual function, as a primary rRNA-binding protein (5) and as a translational repressor of its own synthesis. L1 binds to a specific binding site on its own mRNA, which mimics the specific target site on the 23S rRNA, and thereby down-regulates translation of the entire operon (6,7). L1 consists of two globular domains that are connected by a hinge region (8–11). Two crystal structures were obtained for the L1 protein from *Thermus thermophilus* (TthL1): one in which the protein is unbound and a second in which the protein is bound to the specific binding site on the mRNA (8,12). It was demonstrated that the free L1 displayed a 'closed'

conformation, in which both domains are shifted towards each other (8). In contrast, when TthL1 was bound to rRNA, the individual domains moved away from each other and formed an 'open' conformation (12). Crystal structures of thermophilic archaeal L1 proteins revealed that the L1 ribosomal proteins from *Methanococcus jannaschii* and *Methanococcus thermolithotrophicus* fold into an open conformation even in the absence of RNA, suggesting that the hinge region of thermophilic archaeal L1 proteins is too rigid to allow formation of the closed structure (9,10). Interestingly, crystal structures of L1 and its rRNA-binding site were never obtained in the context of the large ribosomal subunit due to the high flexibility of the L1-binding area on the stalk region (13–16). It was suggested that this flexibility might be caused by ribosomal protein L1, which could actively be involved in E-site tRNA release. Also, ribosomal protein L1 from *E. coli* showed strong RNA chaperone activity *in vitro*, an activity that might be beneficial for facilitating flexibility of rRNA and tRNA (1).

Ribosomal protein L1 from *E. coli* (EcoL1) is structurally and functionally interchangeable with the mesophilic archaeal L1 protein from *Methanococcus vannielii* (MvaL1) and eucaryotic L1 proteins (17,18). Due to the high conservation and the strong RNA chaperone activity observed from EcoL1, we wanted to evaluate whether RNA chaperone activity of L1 is conserved among bacteria, archaea and eukarya.

MATERIALS AND METHODS

Cloning, expression and purification of ribosomal proteins

EcoL1 (1), MjaL1, MvaL1 and TthL1 as well as the MjaL1 mutants (19) were purified as described previously.

A DNA fragment encoding *Sulfolobus solfataricus* (Sso) RPL1 flanked by a 5′-NdeI and a 3′-SapI site was generated by standard PCR with the primers 5′-NdeI-Ss-rpl1AB (5′-TGAAAGAGTCATATGCAAATTGTTGA TAGAAGTAATCTTG-3′) and rpl1AB-Ss-SapI-3′ (5′-TAGAGCGCTCTTCCGCACCTTAACTCTACTTT TACTACCTTACCCATAG-3′) using Sso genomic DNA as template (kindly provided by Udo Bläsi) and cloned into pTWIN1 (New England Biolabs) resulting in pTWIN1-SsoL1. *E. coli* Rosetta (DE3) (Novagen) containing the plasmid pRARE for expression of rarely used tRNAs in *E. coli* was transformed with pTWIN1-SsoL1. Cells were grown at 37°C in LB supplemented with 35 μg/ml chloramphenicol and 100 μg/ml ampicillin. At an optical density of 0.6 at 600 nm the temperature was shifted to 18°C, protein expression was induced by addition of 1 mM IPTG (final concentration) and incubation was continued overnight. Protein was purified using the IMPACT^TM–TWIN System (New England Biolabs). Eluted fractions were analysed *via* SDS–PAGE, pooled, dialysed against Nierhaus buffer 4 (20 mM Tris–HCl pH 7.4; 4 mM MgAc$_2$; 400 mM NH$_4$Cl; 0.2 mM EDTA; 5 mM β-mercaptoethanol) (20) and concentrated. The protein concentration was determined via Bio-Rad protein assay and purity of the protein preparation was checked by SDS–PAGE followed by Coomassie staining.

DNA Fragments encoding human (HsaL1) RPL1, *S. cerevisiae* (SceL1) RPL1 or *C. elegans* (CelL1) RPL1 flanked by a NdeI and a XhoI site were generated by standard PCR using the primer-combinations 5′-NdeI-HsaL1 (5′-GCGTGAGAACATATGAGCAGC AAAGTCTCTCGCG-3′) and HsaL1-XhoI-3′ (5′-ACTG ACTGCTCGAGATATAGGCGCTGGGGCTTGC-3′), 5′-NdeI-SceL1 (5′-ACATATCATCATATGTCTAAGA TCACCTCTTCTCAAGTCAG-3′) and SceL1-XhoI-3′ (5′-ACTGACTGCTCGAGGTACAATCTGAAAGCTG GACCCATG-3′) or 5′-NdeI-CelL1 (5′-TTTCAGGGTCA TATGTCGAAGGTTTCCCGCGAGT-3′) and CelL1-XhoI-3′ (5′-ACTGACTGCTCGAGATAAACTCTTTG TGGCTTTCCCATGG-3′) as well as the templates human cerebral cortex Marathon-Ready cDNA (Clontech), *C. elegans* genomic DNA (kindly provided by M. Glotzer) or yeast genomic DNA, respectively and the PCR products were cloned into pET42a (Novagen) resulting in pET42a-HsaL1, pET42a-SceL1 and pET42a-CelL1. *E. coli* Rosetta (DE3) containing the plasmid pRARE was transformed with pET42a-HsaL1, pET42a-SceL1 or pET42a-CelL1, respectively. Cells were grown at 37°C in LB supplemented with 35 μg/ml chloramphenicol and 100 μg/ml ampicillin. Protein expression was induced at an optical density of 0.6 at 600 nm by addition of 1 mM IPTG (final concentration) and incubation was continued for 2 h. Cells were harvested and sonicated in Nierhaus buffer 4. Inclusion bodies were collected and washed extensively in buffer W1 (20 mM Tris–HCl pH 7.4; 5 mM EDTA, 0.5% Triton X-100) and buffer W2 (20 mM Tris–HCl pH 7.4; 5 mM EDTA). Inclusion bodies were resuspended in denaturing Nierhaus buffer 4 (8 M urea; 20 mM Tris–HCl pH 7.4; 4 mM MgAc$_2$; 400 mM NH$_4$Cl; 0.2 mM EDTA; 5 mM β-mercaptoethanol) incubated with Ni-NTA-Agarose (Qiagen) and applied to a BioRad column. Protein refolding was performed on the column using denaturing Nierhaus buffer 4 with gradually reduced concentration of urea. RPL1 proteins were eluted using Nierhaus buffer 4 supplemented with 250 mM imidazole. Eluted fractions were analysed via SDS–PAGE, pooled and dialysed against Nierhaus buffer 4. Protein concentrations were determined via Bio-Rad protein assay and purity of the protein preparation was checked by SDS–PAGE followed by Coomassie staining.

In vitro transcription

For the *trans*-splicing assay the pTZ18U-tdΔ1-3-derived plasmid H1 (exon1 of the *thymidylate synthase* from T4 phage and 131 nt of the 5′part of the intron) was linearized with SalI and H2 (3′ half of the intron and 23 nt of exon 2) was linearized with BamHI (21). Both constructs were transcribed (and internally labelled) with 40 mM Tris–HCl 7.0, 26 mM MgCl$_2$, 3 mM spermidine, 10 mM DTT, 5 mM ATP, 5 mM GTP, 5 mM UTP, 2.5 mM CTP, α-^{35}S-CTP and T7-RNA polymerase at 37°C for 3 h, followed by DNase digest and purification of the transcripts on a 5% polyacrylamide gel.

For the *cis*-splicing assay plasmid pTZ18U-tdΔP6-2 containing the *thymidylate synthase* intron of T4 phage flanked by short exon sequences (27 nt exon 1, 2 nt exon 2)

was linearized with XbaI (22). Transcriptions were performed under non-splicing conditions with 40 mM Tris–HCl 7.5, 6 mM MgCl$_2$, 10 mM NaCl, 20 mM DTT, 2 mM spermidine, 3 mM ATP, 3 mM GTP, 3 mM UTP, 1 mM CTP, 3 μCi α-^{35}S-CTP and T7-RNA polymerase at 22°C overnight. Transcripts were treated with DNase and purified over a 5% polyacrylamide gel.

For the competition-binding assay, plasmids pMjaL1rH6f.I encoding the 80-nt long 23SrRNA binding site of MjaL1 cloned into a pUC18 vector and plasmid pMvaL1mH5 encoding the 38-nt long minimal mRNA-binding site of *M. vannielii* [Platonova O. (2004); PhD thesis, University of Innsbruck, Austria] were both linearized with SmaI and transcribed using the RNAmaxx *in vitro* transcription kit (Stratagene). Transcripts were purified over an 8% polyacrylamide gel and used in the competition assay.

rRNA competition assay

The transcribed 23S rRNA fragment or the mRNA minimal transcript were incubated for 1 min at 95°C and cooled to 37°C. Binding buffer (50 mM Tris–HCl 7.5, 20 mM MgCl$_2$, 350 mM KCl, 1 mM β-mercaptoethanol) and equimolar amounts of the respective L1 protein were added and were incubated for 15 min at 37°C. Two micromolar final concentration of the pre-bound L1 protein was then utilized in the *trans*-splicing assay.

Trans-splicing assay

The *trans*-splicing assay was performed as previously described (1). Briefly, 200 nM of each H1, H2 transcripts (final concentration 20 nM each) were denatured for 1 min at 95°C and cooled to the respective assay temperature (37, 55 or 60°C). Splicing buffer (4 mM Tris–HCl 7.4, 3 mM MgCl$_2$, 0.4 mM spermidine, 4 mM DTT final concentration), 0.33 pMol ^{32}P-GTP and either the respective ribosomal protein to a final concentration of 2 μM or the same volume of protein storage buffer were added. Splicing was stopped by the addition of 40 mM EDTA, 300 μg/ml tRNA, the samples were extracted with phenol/CHCl$_3$, precipitated and analysed on 5% poly-acrylamide gels. Gels were analysed with PhosphorImager and the software ImageQuant. The relative splicing rates were obtained by the formula (nx – n37)/(n55 – n37), whereas nx being the relative splicing rate to be determined, n55, the relative splicing rate at 55°C in the presence of protein storage buffer and n37, the relative splicing rate at 37°C in the presence of protein storage buffer. Protein storage buffer slightly inhibits *trans*-splicing. All L1 proteins were higher concentrated except for the eukaryal proteins, which were low concentrated so that also high amounts of protein storage buffer had to be added to the positive control, which resulted in a decrease of *trans*-splicing at 55°C. Therefore the relative *trans*-splicing rates of the eukaryal proteins should be compared internally but not in respect to the *trans*-splicing rate of EcoL1.

Cis-splicing assay

Cis-splicing was performed as previously described (23). Briefly, 0.5 pMol ^{35}S-body-labelled precursor RNA (precursor RNA was a modified *thymidylate synthase* gene of T4 phage containing short exon sequences—27 nt exon 1, 2 nt exon 2) were denatured for 1 min at 95°C, cooled to 37°C and splicing was started by the addition of splicing buffer (50 mM Tris–HCl 7.3, 5 mM MgCl$_2$, 0.4 mM spermidine) and 0.5 mM GTP. The respective protein was added to a final concentration of 2 μM. The reaction was stopped with 40 mM EDTA and 300 μg/ml tRNA. Samples were extracted with phenol/CHCl$_3$, precipitated and analysed on 5% polyacrylamide gels. Gels were analysed with PhosphorImager and the software ImageQuant.

Filter-binding assay

In vitro transcribed *trans*-splicing substrates H1 and H2 were dephosphorylated, labelled with γ-^{32}P-ATP and purified over a G50 column. Equimolar amounts of H1 and H2 were pre-heated at 95°C for 1 min and cooled to 37°C. Splicing buffer (4 mM Tris–HCl 7.4, 3 mM MgCl$_2$, 0.4 mM spermidine, 4 mM DTT final concentration) and the respective protein at various concentrations were added and the reactions were incubated for 20 min at 37°C. Samples were then applied to nitrocellulose filters, the filters were washed twice with 1 ml 1 × splicing buffer, dried for a short time and analysed in a scintillation counter. The curves were fitted to the equation for non-cooperative binding $[ML_n] = [M_0]/(1 + (K_{1/2}/[L])^{nH})$ ($[M_0]$ = the RNA concentration, $K_{1/2}$ = the dissociation constant, $[L]$ = the protein (ligand) concentration and nH = the Hill coefficient).

RESULTS

RNA chaperone activity of rpL1 is conserved in all three domains of life

The previously described *trans*-splicing assay is a convenient and reliable method to test RNA chaperone activity of isolated proteins *in vitro* (1). The assay is based on ribozyme cleavage of the self-splicing group I intron in the *thymidylate synthase* gene of T4 phage (21). Two separate constructs (H1, H2) are *in vitro* transcribed and internally labelled with α-^{35}S-CTP (H1, which contains 549 nt of exon1 and 131 nt of the 5′-part of the intron and H2 containing 147 nt of the 3′-part of the intron and 23 nt of exon2). During *trans*-splicing the two RNAs anneal, splicing is induced by addition of ^{32}P-guanosine and the first step of splicing (the formation of ^{32}P-guanosine ligated to the 5′-part of the intron) is monitored. Typically, we follow the reaction at 37°C, where *trans*-splicing is not detectable in the absence of auxiliary factors like RNA chaperones. Additional weak bands that are visible after long incubation times (e.g. after 60 min) correspond to splicing intermediates and cryptic splice products (24). Strong additional bands (labelled with * in Figure 1B) correspond to intron oligomers, which result from transesterification reactions of excised intron
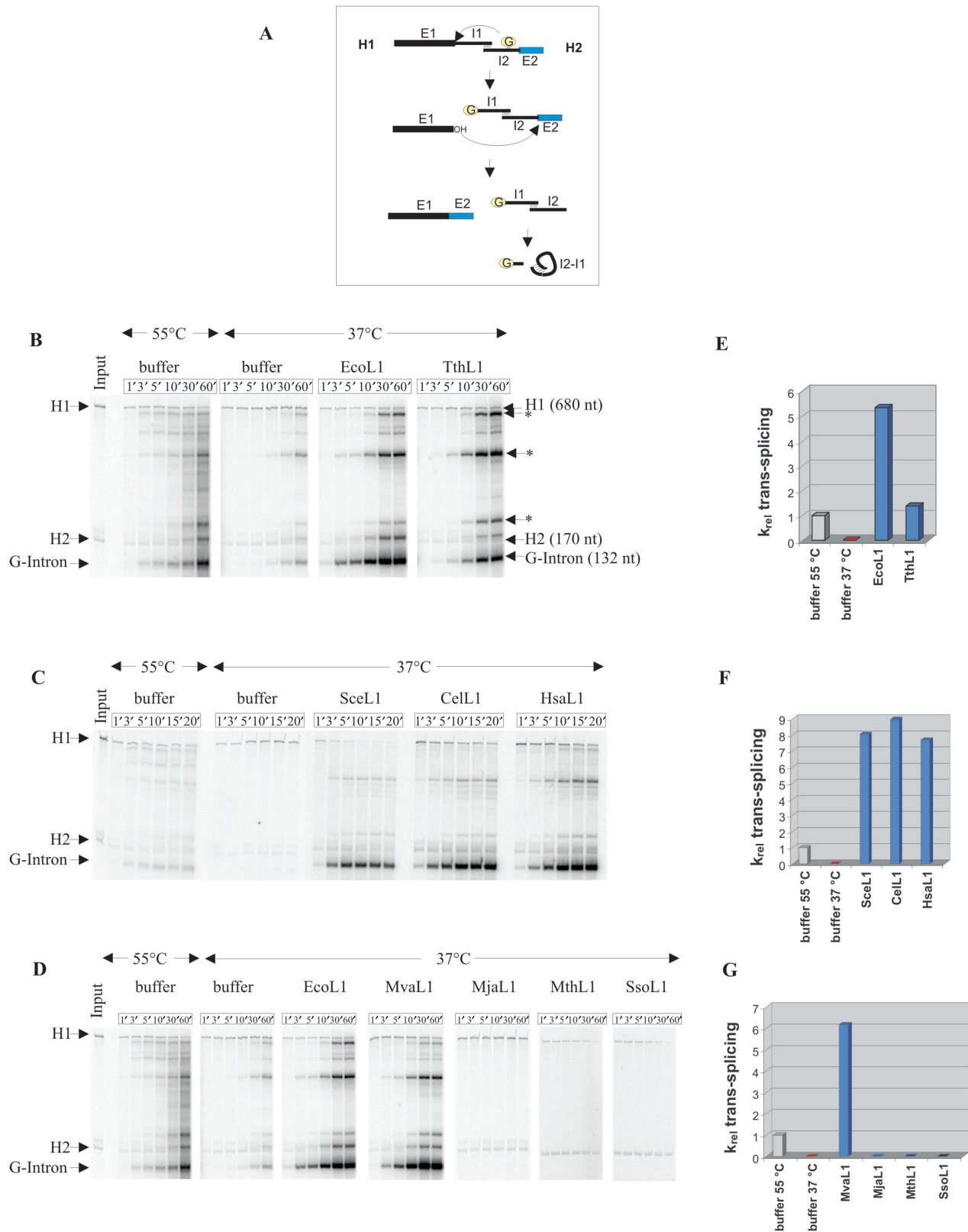
Figure 1. RNA chaperone activity is conserved among all three domains of life. **(A)** The *trans*-splicing scheme: RNAs H1 and H2 are annealed and splicing is started by the addition of the guanosine cofactor. E1 (exon 1), I1 (5′-part of the intron), I2 (3′-part of the intron) and E2 (exon 2) are labelled. **(B–D)** Representative splicing gels in the presence of various L1 proteins (final concentration 2 μM). H1 corresponds to exon 1 of the *thymidylate synthase* gene of phage T4 and the 5′-part of the intron, H2 corresponds to the 3′-part of the intron and exon 2 and **G–I** corresponds to the splice-product (guanosine ligated to the 5′-intron part). Bands labelled with ∗ correspond to intron oligomers. Figure 1B shows *trans*-splicing in the presence of bacterial L1 proteins from *E. coli* (third set of reactions) and from *T. thermophilus* (fourth set). The first set shows the positive control of *trans*-splicing in the absence of additional protein at 55°C and the second set shows the negative control *trans*-splicing in the absence of protein at 37°C. Figure 1C shows a typical *trans*-splicing assay performed in the presence of L1 from *S. cerevisiae* (third set), *C. elegans* (fourth set) and *H. sapiens* (fifth set). Figure 1D shows *trans*-splicing in the presence of EcoL1 (third set) and in the presence of archaeal L1 proteins from *M. vannielii* (fourth set), *M. jannaschii* (fifth set), *M. thermolithotrophicus* (sixth set) and *S. solfataricus* (seventh set). Experiments were performed at least twice. Figure **1E–G** shows the relative *trans*-splicing rates that were obtained by the formula $(nx - n37)/(n55 - n37)$, whereby nx is the relative splicing rate to be determined, $n55$, the relative splicing rate at 55°C in the presence of protein storage buffer, and $n37$, the relative splicing rate at 37°C in the presence of protein storage buffer.

sequences and other excised intron sequences that include the 5′-added ^{32}P-labelled guanosine (25).

The *trans*-splicing assay was performed at 55°C (positive control) and 37°C (negative control) both in the absence of proteins. Ribosomal proteins were then added to the reaction at 37°C to a final concentration of 2 μM. Figure 1A shows a schematic representation of the *trans*-splicing assay. Figure 1B–D show three representative *trans*-splicing gels and Figure 1E–G show the quantification charts, where relative *trans*-splicing rates were obtained by the formula $(nx – n37)/(n55 – n37)$ with n55 and n37 corresponding to *trans*-splicing rates at 55 and 37°C, respectively and nx being the *trans*-splicing rate in the presence of the respective protein. Figure 1B shows *trans*-splicing in the presence of two bacterial L1 proteins from the mesophilic bacterium *E. coli* (EcoL1) and the thermophilic *Thermus thermophilus* (TthL1). Both show significant enhancement of *trans*-splicing although TthL1 has reduced RNA chaperone activity at 37°C (26% chaperone activity compared to EcoL1).

We also tested RNA chaperone activity of eucaryotic ribosomal L1 proteins from *Saccharomyces cerevisiae* (SceL1), *Caenorhabditis elegans* (CelL1) and *Homo sapiens* (HsaL1). Figure 1C and F show the *trans*-splicing assays and their quantification: all three eucaryotic L1 proteins significantly increase *trans*-splicing at 37°C.

Finally, we tested archaeal rpL1 in the *trans*-splicing assay (Figure 1D). Interestingly, ribosomal protein L1 from the mesophilic archaea *Methanococcus vannielii* (MvaL1) shows even higher RNA chaperone activity than EcoL1 (6–7 times higher than positive control for MvaL1 compared to 5–6 times higher than positive control with EcoL1). In contrast, none of the three L1 proteins tested, which were isolated from the thermophilic archaea *Methanococcus jannaschii* (MjaL1), *Methanococcus thermolithotrophicus (*MthL1*)* and *Sulfolobus solfataricus* (SsoL1), showed RNA chaperone activity. This result was quite surprising, taking into account that the three L1 proteins from the Methanococci species *M. vannielii*, *M. jannaschii* and *M. thermolithotrophicus* show more than 70% sequence identity at the amino acid level.

Analysis of archaeal thermophilic ribosomal L1 proteins: alternative RNA chaperone assays and different temperature and salt conditions

- *Thermophilic, archaeal L1 proteins do not exhibit RNA chaperone activity in the cis-splicing assay*: We previously observed that some proteins (e.g. the human La protein) that show strong RNA chaperone activity in other RNA chaperone assays (strand annealing assay, *cis*-splicing assay) do not necessarily show RNA chaperone activity in the *trans*-splicing assay (23). We therefore wanted to test the possibility that the thermophilic archaeal L1 proteins display RNA chaperone activity in an alternative assay and chose to use the previously described *cis*-splicing assay (22). In this assay, splicing of the *in vitro* transcribed group I intron of the *thymidylate synthase* gene, which is flanked by short exon sequences (27 nt of exon 1 and
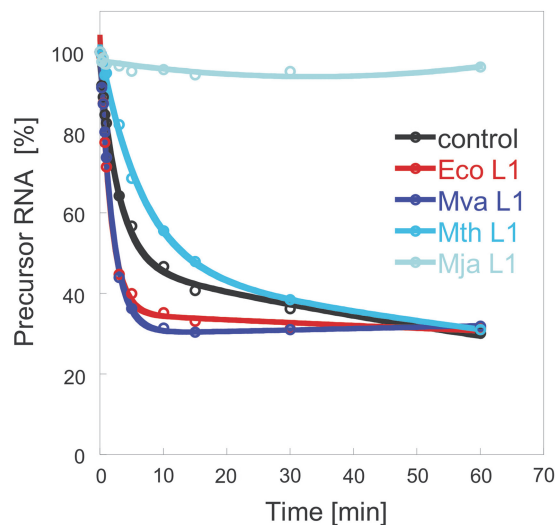
	k_{obs} [min]
control	**0.348 (+/− 0.144)**
EcoL1	**0.760 (+/− 0.235)**
MvaL1	**0.466 (+/− 0.038)**
MthL1	**0.245 (+/− 0.099)**
MjaL1	**< 0.001**

Figure 2. RNA chaperone activity of EcoL1 and MvaL1 is observed in the *cis*-splicing assay. The chart shows the decrease of precursor RNA (*y*-axis) of a self-splicing group I intron in time (*x*-axis). Either precursor RNA alone (black line) or the precursor RNA in the presence of 2 μM EcoL1 (red line), MvaL1 (dark-blue line), MthL1 (blue line) or MjaL1 (light-blue line) was subjected to *cis*-splicing. Results were obtained from at least three independent experiments and average *cis*-splicing rates (k_{obs} per minute) are summarized.

2 nt of exon 2), is monitored. Due to the short exon sequences folding of the *in vitro* transcribed construct is reduced and as a consequence splicing is also reduced. In the *cis*-splicing assay we measure the decrease of precursor RNA at 37°C after the addition of exogenous guanosine. Figure 2 shows the quantification of precursor RNA in the absence of proteins (control, black line) and in the presence of 2 μM EcoL1 (red line), MvaL1 (dark-blue line), MthL1 (blue line) and MjaL1 (light-blue line) proteins. Both EcoL1 and MvaL1 increase *cis*-splicing. However in each case only 65% of molecules undergo splicing, the same as in the absence of added protein, suggesting that 35% of the precursor RNAs are severely misfolded and cannot be refolded even in the presence of RNA chaperones. MthL1 protein does not increase *cis*-splicing. Surprisingly, while MjaL1 behaved similarly to MthL1 in the *trans*-splicing assay, it completely inhibits *cis*-splicing. We also tested L1 from the thermophilic archaeal SsoL1 in the *cis*-splicing assay and SsoL1 acts similarly to MthL1, not inhibiting *cis*-splicing but show no RNA chaperone activity (data not shown). These results are largely consistent with the *trans*-splicing data, as both EcoL1 and

MvaL1 increase splicing while the thermophilic archaeal L1 proteins do not.

- *Thermophilic, archaeal ribosomal L1 proteins do not display RNA chaperone activity at elevated temperatures nor at high concentrations of monovalent or divalent metal ions*: The lack of RNA chaperone activity of thermophilic archaeal L1 proteins could be due to the temperature difference between the assay conditions (37°C) and the optimal growth temperatures of the respective organisms (85°C for *M. jannaschii* and 65°C for *M. thermolithrophicus*). Therefore, we increased the assay temperature of the *trans*-splicing assay to 55 and 60°C. We wanted to reduce the temperature discrepancy between optimal growth of the archaea and assay conditions without melting the 3D structure of the group I intron. It was shown previously that the *td* group I intron 3D structure starts to melt at 55°C and that at this temperature splicing occurs most efficiently (26,27). Figure 3 shows a representative *trans*-splicing gel: *trans*-splicing occurs efficiently in the absence of proteins at 55°C and at 60°C. In both control lanes, we added the same volume of protein storage buffer to exclude the possibility that the storage buffer by itself influences the reaction. The presence of either EcoL1 or MvaL1 significantly increases *trans*-splicing at 55°C, although the growth optimum of both species is 37°C. Due to the elevated temperature, background bands corresponding to splicing intermediates and intron oligomers increase. The presence of MthL1 and MjaL1 proteins at 55°C did not increase *trans*-splicing and in fact, both proteins inhibit *trans*-splicing relative to the control at elevated temperatures, as MjaL1 does in the *cis*-splicing assay at 37°C. Next, we tested *trans*-splicing at 60°C in the presence of MthL1, as the optimal growth temperature of this thermophilic archaea is 65°C. However, even at 60°C MthL1 does not show any RNA chaperone activity in the *trans*-splicing assay and in fact inhibits *trans*-splicing at this temperature.

To test whether high monovalent ion concentrations might modulate the activity of MjaL1 from splicing inhibition to chaperoning, we performed *trans*-splicing assays at 55°C with the standard splicing buffer (see 'Materials and Methods' section) but added either high-monovalent or high-divalent salts as indicated (supplementary Figure 1). However, high monovalent ion concentrations (250 mM KCl, 500 mM KCl) inhibit splicing of the *td* group I intron in the absence of added protein. Thus, although the splicing inhibition resulting from the high-ion concentrations is not alleviated in the presence of MjaL1 (Supplementary Figure 1), we cannot rule out the possibility that MjaL1 or other L1 proteins from thermophilic archaea might exhibit RNA chaperone activity under normal physiological conditions. We also wanted to know whether the mechanism of MjaL1 inhibition of group I intron splicing could be due to an effect on the availability of Mg^{++} ions necessary for splicing catalysis (28). To test this possibility, we performed *trans*-splicing assays in the presence of 10 mM $MgCl_2$ with or without MjaL1 (see Supplementary Figure 1). While *trans*-splicing proceeds at 55°C in the presence of 10 mM $MgCl_2$, the presence of MjaL1 (2 μM) still leads to complete splicing inhibition. This result suggests that MjaL1 does not inhibit splicing by sequestering Mg^{++} ions required for catalysis (see Supplementary Figure 1).
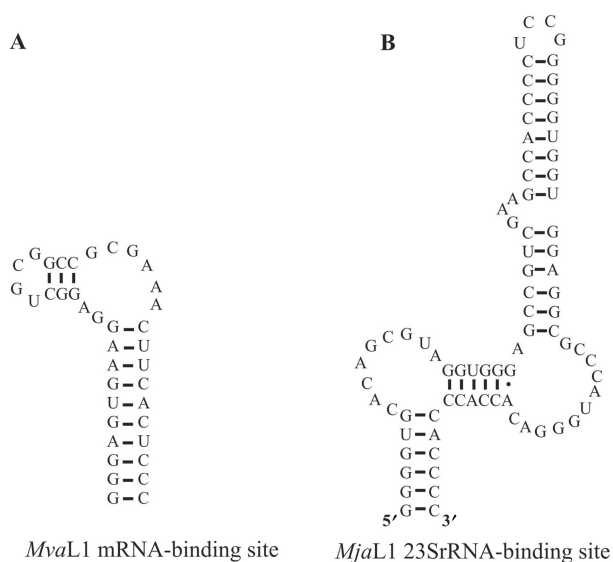
- *Inhibition of splicing by thermophilic, archaeal rpL1*: It is most likely that inhibition of *trans*-splicing also occurs at 37°C in the presence of MjaL1 and MthL1, and indeed when we prolonged the measurement times for *trans*-splicing at 37°C in the absence of protein, we observed low *trans*-splicing, which is inhibited in the presence of MjaL1, MthL1 or SsoL1. MjaL1, in addition, completely inhibits *cis*-splicing, which is inhibited by neither MthL1 nor SsoL1. To our knowledge, no other protein has been shown to inhibit group I intron splicing *in vitro*. Thus, ribosomal protein L1 from *M. jannaschii* is particularly noteworthy.
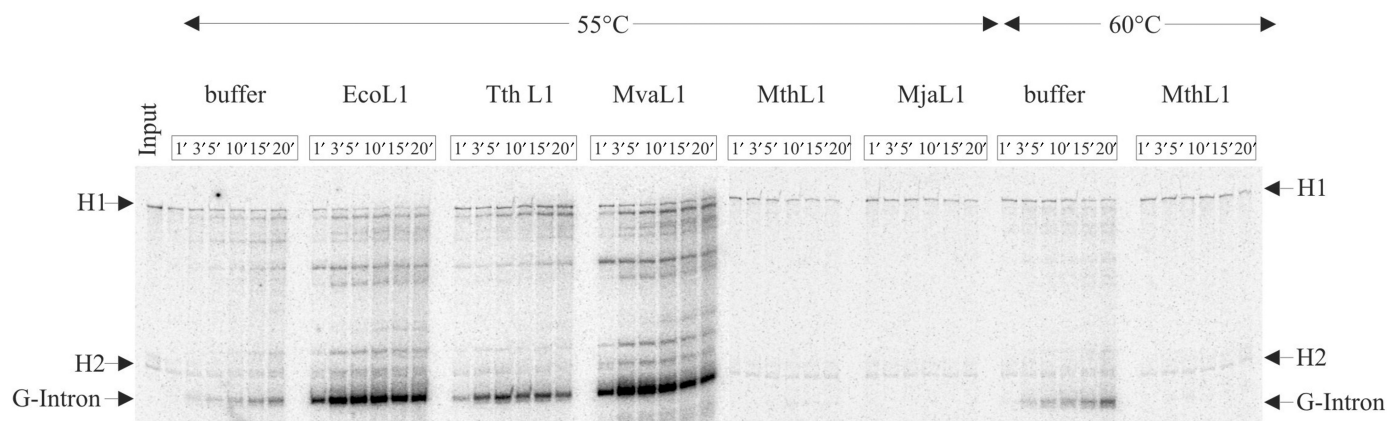
Figure 3. Thermophilic archaeal L1 proteins inhibit *trans*-splicing also at elevated temperatures. Figure 3 shows a typical *trans*-splicing gel: *trans*-splicing was performed in the presence of 2 μM proteins at either 55°C (set 1–6) or at 60°C (set 7, 8). The first set of experiments shows the control *trans*-splicing in the absence of protein at 55°C and the seventh set of experiments shows *trans*-splicing in the absence of protein at 60°C. Experiments were repeated at least three times.

Loss of RNA chaperone activity when rpL1 is bound to ribosomal RNA or mRNA

So far, we tested RNA chaperone activity of free/unbound rpL1 in the various chaperone assays. Thus, we were interested if RNA chaperone activity is modulated when ribosomal protein L1 is pre-bound to either its rRNA or its mRNA target sequence. It was recently shown that the rRNA and mRNA rpL1 target sequences of *M. jannaschii* can be minimized to an 80-nt long and a 38-nt long stem-loop sequence, respectively, which contain an asymmetric loop (Figure 4), without losing binding activity. Furthermore, rRNA and mRNA binding sites are highly conserved in bacteria and archaea so that they are interchangeable between species (29,30). In this assay, we used an 80-nt long *in vitro* transcribed *M. jannaschii*

23SrRNA construct or the *in vitro* transcribed mRNA minimal binding-site of *M. vannielii* (38 nt) (Figure 4) and incubated EcoL1 in a 1:1 molar ratio together with the respective rRNA or mRNA (see 'Materials and Methods' section). The pre-bound complex was then subjected to the *trans*-splicing assay. Figure 5 shows a typical *trans*-splicing gel: EcoL1 protein significantly increases *trans*-splicing at 37°C (Figure 5; third set of reactions) and the ribosomal RNA alone does not influence *trans*-splicing (Figure 5; sixth set). When EcoL1 is pre-bound to the 23S rRNA-binding site the RNA chaperone activity is lost, suggesting that either the inner surfaces of domain I and domain II of ribosomal protein L1, which are now occupied by rRNA are involved in RNA chaperoning, or that by binding of EcoL1 to the rRNA conformational rearrangements take place, which result in loss of RNA chaperone activity. Figure 5, fifth set of assays, shows EcoL1 pre-bound to a control RNA (firefly luciferase RNA of similar length). RNA chaperone activity is reduced in this experiment but not as drastically as in the case of L1 being pre-bound to the rRNA, suggesting that the alleviation of the RNA chaperone activity depends on the affinity of the RNA–protein interaction. The same reduction in *trans*-splicing is observed when L1 is pre-bound to its mRNA target site (data not shown). mRNA binding is an order of magnitude less than binding to rRNA (31) and therefore the suppression of the RNA chaperone activity by pre-binding to mRNA is less severe.

Loss of splicing-inhibition of MjaL1 bound to ribosomal RNA

We next asked whether pre-binding of MjaL1 to its 23S rRNA binding-site affects *trans*-splicing. The *trans*-splicing assay was performed at 55°C, in order to bring the temperature more in line with the optimal growth temperature. MjaL1 was incubated for 15 min together with *in vitro* transcribed 23SrRNA and 2 µM final protein–RNA complex was subjected to the *trans*-splicing assay. Interestingly, pre-binding of MjaL1 to the 23S rRNA-binding site alleviated the inhibitory effect (Supplementary Figure 2) and splicing occurred at levels
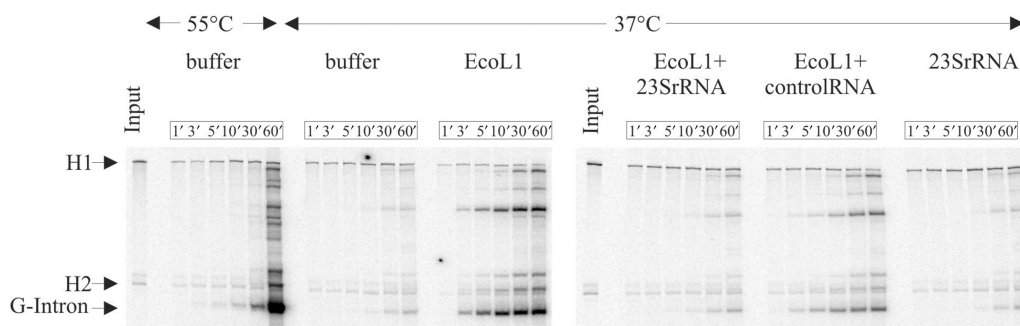

Figure 4. mRNA and 23SrRNA target sites for binding of L1 protein. (A) Secondary structure of mRNA fragment and (B) of 23S rRNA fragment that were used in the RNA competition assay are shown. The mRNA target derived from *M. vannielii* and the 23S rRNA transcript from *M. jannaschii*.


Figure 5. RNA chaperone activity is suspended when EcoL1 is pre-bound to RNA. Figure 5 shows a typical *trans*-splicing gel with the positive control, splicing at 55°C in the absence of protein (first set), splicing in the absence of protein at 37°C (second set; negative control) and in the presence of 2 µM EcoL1 (third set). EcoL1was also pre-bound to either the *in vitro* transcribed and 80 nt in length Mja 23SrRNA-binding site (fourth set) or the firefly luciferase RNA (fifth set). Two micromolar of each pre-bound complex were then used in the *trans*-splicing experiment. The sixth set of splicing shows *trans*-splicing in the presence of the Mja23SrRNA fragment only.

comparable to the experiment performed in the absence of MjaL1. We also tested a MjaL1 point mutant where threonine$_{204}$ is substituted with phenylalanine, which lost its high affinity for the specific mRNA and rRNA target sites. However, MjaL1T204F still inhibits *trans*-splicing at 55°C and the inhibitory effect disappears in the presence of mRNA (Supplementary Figure 2).

We also demonstrate that MjaL1 is capable of binding to its 23SrRNA and mRNA target sites, as was shown previously (31). Supplementary Figure 3 shows filter-binding assays of MjaL1 to 23SrRNA, to mRNA and to 16SrRNA, illustrating that indeed the purified protein binds to its target RNAs (23SrRNA and mRNA) but not to 16SrRNA.

Concentration dependence of RNA chaperone activity and splicing inhibition

In the *trans*-splicing experiments we always used 2 µM final protein concentration for evaluation of RNA chaperone activity. We were interested in whether RNA chaperone activity in the presence of EcoL1 and the splicing inhibition in the presence of MjaL1 are concentration dependent. Therefore, we performed *trans*-splicing experiments at 37°C with various protein concentrations of EcoL1 (10, 50, 100, 200, 500 nM, 1 µM) and *trans*-splicing experiments at 60°C with various protein concentrations of MjaL1 (10, 50, 100, 200, 300, 400, 500 nM, 1 µM). Figure 6 shows the protein concentrations plotted versus the relative *trans*-splicing rates. Note that *trans*-splicing experiments with EcoL1 and MjaL1 were
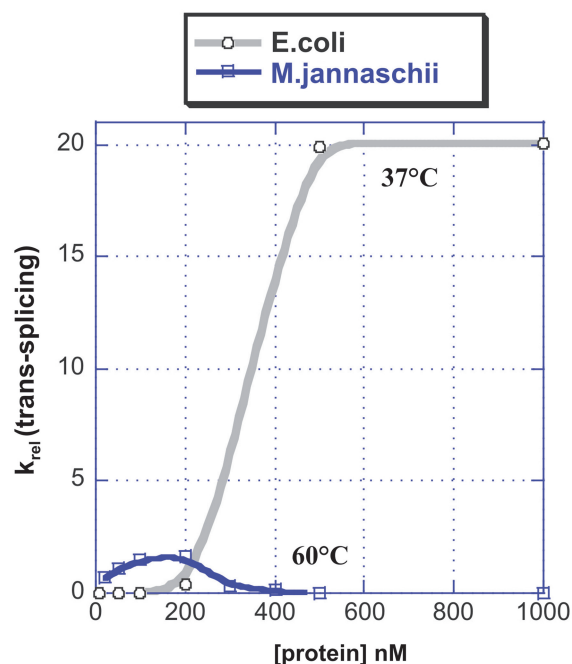
Figure 6. Chaperone activity and splicing inhibition are concentration dependent. The chart shows relative *trans*-splicing rates in the presence of varying concentrations of EcoL1 performed at 37°C (gray line). The blue line shows relative *trans*-splicing rates measured at 60°C in presence of varying concentrations of MjaL1. The *y*-axis shows relative *trans*-splicing rates and the *x*-axis shows protein concentration in nM.

performed at different temperatures (37 and 60°C, respectively). We observed that enhancement of *trans*-splicing in the presence of EcoL1 occurs at a protein concentration of 450 nM and splicing inhibition in the presence of MjaL1 appears at around 200 nM final protein concentration. It is also interesting that the enhancement of *trans*-splicing at lower protein concentrations in the presence of EcoL1 seems to be higher than at 2 µM final concentration, suggesting that there is an RNA chaperone activity maximum at around 1 µM protein concentration.

Mutational analysis of MjaL1: RNA chaperone activity versus RNA binding

Ribosomal proteins are strong RNA-binding proteins with binding constants to mRNA or rRNA-binding sites in the low nanomolar and picomolar ranges. The thermophilic archaeal L1 proteins bind 2 orders of magnitude stronger to their rRNA target sequences than EcoL1 (30,31). We suspected that this strong RNA-binding activity of MjaL1 leads to the inhibitory effect, which we observe during *cis*- and *trans*-splicing *in vitro* and that a loss of binding might result in a gain of RNA chaperone activity. Therefore, we tested RNA chaperone activity of MjaL1 mutants (Figure 7A and B), which showed a significant reduction in mRNA and rRNA binding (data not shown). The MjaL1 mutants had either single amino acid substitutions in domain I (T204F, M205D, M205F), in domain II (R126A) or at the hinge region (R54K) (Figure 7C). None of the MjaL1 mutants, however, lost the inhibitory effect on *cis*- or *trans*-splicing nor did they gain RNA chaperone activity (Figure 7A and B). *Trans*-splicing was repeated at elevated temperature (60°C) with all previously discussed mutant L1 proteins and they all inhibited *trans*-splicing to the same extent as they did for *trans*-splicing at 37°C (data not shown), suggesting that the single amino-acid substitutions are not sufficient to alter the inhibitory effect on splicing.

Next, we tested whether MjaL1 or one of the MjaL1 mutants binds significantly stronger to the *trans*-splicing substrates (H1, H2) compared to EcoL1. We performed filter-binding assays using 0.5 pMol H1–H2 substrates that were pre-heated and cooled to 37°C to allow folding. Then, L1 protein was added and filter-binding assays were performed. Salt conditions were chosen to match the conditions of the *trans*-splicing assay (see 'Materials and Methods' section). Figure 8 shows the graph where the relative amount of bound *trans*-splicing substrates H1 and H2 is blotted versus the protein concentration. The curves were fitted with the equation $[ML_n] = [M_0]/(1 + (K_{1/2}/[L])^{nH})$ and the dissociation constant $K_{1/2}$ was determined (Figure 8). MjaL1 showed a $K_{1/2}$ of about 200 nM, both mutants MjaL1T204F and MjaL1R54K showed $K_{1/2}$ of 300 to 500 nM and EcoL1 bound with $K_{1/2}$ of 500 nM. These data demonstrate that under the *trans*-splicing conditions binding of the various L1 proteins to the *trans*-splicing substrates does not differ significantly, suggesting that strong binding of MjaL1 is not the reason for splicing inhibition.
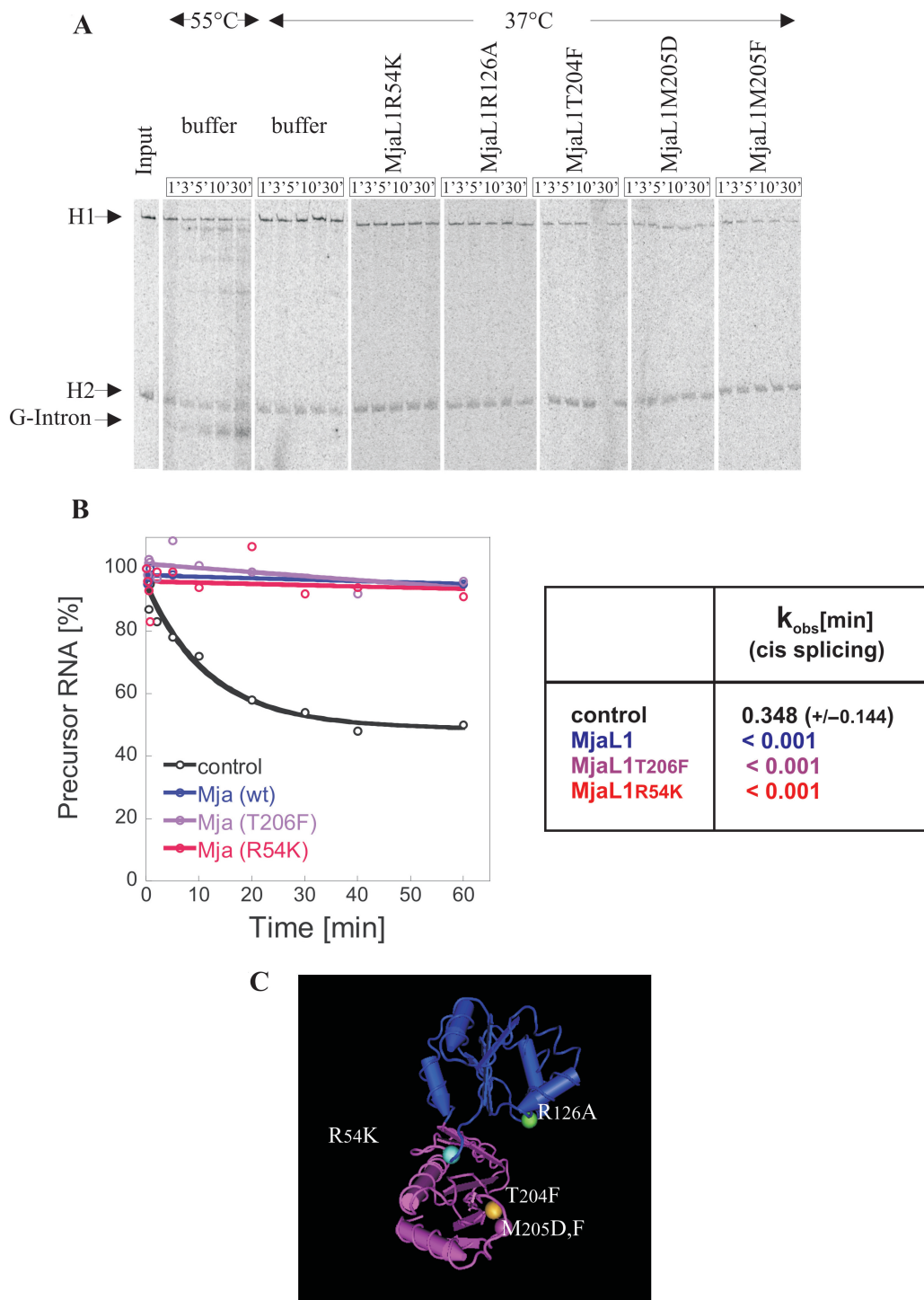
Figure 7. MjaL1 point mutants in both domains and the hinge region do not result in altered splicing inhibition in neither *cis*- nor *trans*-splicing assays. (**A**) *M. jannaschii* mutants with point mutations in domain I (MjaL1T204F, MjaL1M205D, MjaL1M205F), in domain II (MjaL1R126A) or in the hinge region (MjaL1R54K) were tested at a final concentration of 2 μM in the *trans*-splicing assay. (**B**) Both mutants MjaL1T204F and MjaL1R54K were also tested in the *cis*-splicing assay at a final concentration of 2 μM. Average *cis*-splicing rates (k_{obs} per minute) are summarized next to the graph. (**C**) Mutations on the MjaL1 protein are indicated. The structure was derived from (9) and the point mutations were highlighted using the program Cn3D.

DISCUSSION

One-third of all large ribosomal subunit proteins from *E. coli* have RNA chaperone activity *in vitro* (1). We were interested in whether this activity is a general phenomenon for ribosomal proteins and consequently might play an important role during assembly and/or translation. We chose ribosomal protein L1, which showed strong RNA chaperone activity in the *trans*-splicing assay and

	$K_{1/2}$
MjaL1	189.7 (+/−41.6)
Mja[T204F]L1	494.6 (+/−43.1)
Mja[R54K]L1	384.1 (+/−94.4)
EcoL1	541.6 (+/−26.5)
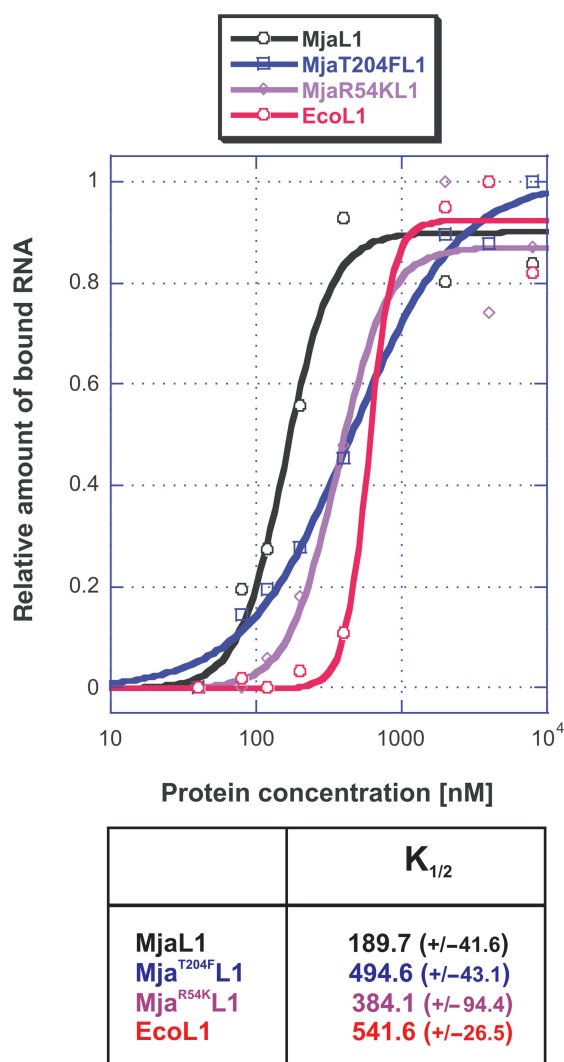
Figure 8. MjaL1 and mutants bind with similar affinity to the *trans*-splicing substrates like EcoL1. Filter-binding assays were performed at various protein concentrations in the presence of MjaL1, MjaL1T204F, MjaL1R54K and EcoL1. Radioactively labelled H1 and H2 substrates were pre-folded and incubated with different protein-concentrations for 20 min at 37°C. Filter-binding assays were performed and the remaining radioactivity on the filter was measured by a scintillation counter. The relative amount of bound RNA (*y*-axis) was blotted against the respective protein concentration (*x*-axis). The table below summarizes the calculated $K_{1/2}$ values and standard deviations obtained from three independent experiments.

which has homologues in all three domains of life, to evaluate whether RNA chaperone activity is conserved. We cloned, over-expressed and purified bacterial, eukaryal and archaeal L1 proteins, tested them in the *trans*- and *cis*-splicing assays and found that RNA chaperone activity was indeed present in a thermophilic, bacterial L1 homologue (*Thermus thermophilus*), in eukarya (*Saccharomyces cerevisiae, Caenorhabditis elegans, Homo sapiens*) and in mesophilic archaea (*M. vannielii*), suggesting that RNA chaperone activity of ribosomal proteins might be an important feature. Interestingly, RNA chaperone activity of the thermophilic bacterial TthL1 was five times lower than

the mesophilic eubacterial EcoL1 RNA chaperone activity. This is consistent with the fact that RNA misfolding is more prominent in the cold, suggesting that thermophilic proteins might have reduced RNA chaperone activity (optimal growth temperature of *T. thermophilus* is 70–75°C). Table 1 summarizes the results and compares RNA chaperone activity in the *trans*- and *cis*-splicing assays with growth temperature of the respective organism. All mesophilic organisms show strong RNA chaperone activity but thermophilic species have either reduced or no RNA chaperone activity (Table 1). In line with this, it was shown that *T. thermophilus* ribosomes have a reduced translation rate at 45°C (25–30% of the maximal rate determined for *E. coli* ribosomes at 37°C) (32).

Thermophilic, archaeal L1 proteins (*M. jannaschii, M. thermolithotrophicus, S. solfataricus*) did not accelerate *trans*-splicing, rather they even inhibited *trans*-splicing at 37°C, at 55°C and at 60°C. Furthermore, MjaL1 inhibited *cis*-splicing. These results were surprising, especially when taking into account that the mesophilic archaeal *M. vannielii* L1 protein, which shares 70% amino acid identity with MjaL1 and MthL1 proteins, has strong RNA chaperone activity. The observation that the thermophilic L1 proteins also do not promote *trans*-splicing at elevated temperatures rules out the possibility that our assay conditions do not provide the appropriate temperature conditions. Furthermore, we showed that the splicing inhibition is very likely not due to stronger binding of MjaL1 nor is it due to sequestering of Mg^{++} ions by MjaL1 required for splicing catalysis. A pre-liminary mutational analysis of MjaL1 with point muta-tions in both domains and within the hinge region did not result in alteration of the inhibitory effect. The majority of these MjaL1 point mutants had lost their specific binding affinity to their target substrates (mRNA, rRNA) but non-specific binding to the group I intron was not altered (Figure 8). Many proteins with specific RNA-binding sites display non-specific RNA chaperone activity. RNA chaperone activity might result from two different mechanisms of interaction with RNA. Before high-affinity binding occurs, the proteins might undergo non-specific interactions inducing conformational changes in the RNA in search of the optimal binding substrate. Recently such a dual mechanism was demonstrated for the group I intron splicing factor CBP2 via single molecule FRET (33). The RNA chaperone activity of L1 might also originate from initial non-specific RNA contacts, which chaperone the folding of its binding substrate. Once the RNA is bound, L1 no longer undergoes non-specific binding. The inhibitory effect of MjaL1 might result from an imbalance between non-specific and specific protein–RNA interac-tions. It has previously been shown for the *E. coli* protein StpA that its RNA chaperone activity is detrimental to folding if the structural stability of the RNA is already low (34), suggesting that this first non-specific interaction of the protein with the RNA might also negatively influence folding. In this line, it has been shown for the CBP2 protein, which also has RNA unfolding activity, that at high concentrations of protein (5–8-fold excess over the RNA concentration) splicing of the bI5 intron

Table 1. Results from both the *trans*-splicing and the *cis*-splicing assays performed in the presence of various L1 proteins from bacteria (first two rows) from archaea (rows 3–6) and from eukarya (rows 7–9)

	Optimal growth temperature (°C)	Activity in *trans*-splicing assay	Activity in *cis*-splicing assay
Escherichia coli L1 (EcoL1)	37	++	+
Thermus thermophilus L1 (TthL1)	85	+	n.d.
Methanococcus vannielii L1 (MvaL1)	37	++	+
Methanococcus thermolithotrophicus L1 (MthL1)	65	Inhibition	−
Methanococcus jannaschii L1 (MjaL1)	85	Inhibition	Inhibition
Sulfolobus solfataricus L1 (SsoL1)	75–85	Inhibition	−
Saccharomyces cerevisiae L1 (SceL1)	28	++	n.d.
Caenorhabditis elegans L1 (CelL1)	25	++	n.d.
Homo sapiens L1 (HsaL1)	37	++	n.d.

Note: All assays were performed in the presence of $2\,\mu M$ protein. Column 2 lists the optimal growth temperature of each organism. '+' or '++' means positive effect on splicing (RNA chaperone activity), '−' means no effect, 'n.d.' means not determined and 'inhibition' stands for inhibition of splicing.

occurs at a reduced rate (35). An increase in protein concentration also resulted in a switch from specific to unspecific RNA–protein interactions (33). We propose that the inhibitory effect of MjaL1 might be due to this unspecific protein–RNA interaction and might influence folding. RNA folding inhibition is a new phenomenon that has not yet been analysed mechanistically and we are currently studying this inhibitory mechanism in depth.

When we tested L1 proteins that were pre-bound to the rRNA-binding site for RNA chaperone activity, we observed that first, RNA chaperone activity of EcoL1 was suspended and second, that the splicing inhibition of MjaL1 was lost. These results suggest that either the bound RNA molecule blocks the site, which encompasses the RNA chaperone activity, or the RNA binding event might lead to structural changes within L1, which then result in loss of RNA chaperone activity. Crystal structures of various L1 proteins were solved: the crystal structures of MjaL1 and MthL1 proteins in solution showed that domain I and domain II were positioned far away from each other resulting in an 'open' conformation of the protein (9,10). MjaL1 bound to its mRNA also captured the 'open' conformation (30). In contrast, in the crystal structure of the thermophilic bacterium *T. thermophilus* both domains were folded up resulting in a 'closed' conformation (8). TthL1, in contrast to the archaeal L1 proteins (MjaL1, MthL1 and SsoL1), showed RNA chaperone activity in the *trans*-splicing assay. Interestingly, in the crystal structure of TthL1 bound to its mRNA, the protein adopts the open conformation (12). It was suggested that all ribosomal L1 proteins adopt the open conformation when they are bound to RNA. Thermophilic archaeal L1 proteins were suggested to be more rigid and only adopt the open conformation. The open structure of the protein might then present an RNA-binding surface and interact non-specifically with any RNA molecule. We suggest that a shift between the proportion of specific to non-specific interactions of a protein might result in a shift between RNA chaperone activity and folding inhibition.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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