RNA chaperone activity of large ribosomal subunit proteins from *Escherichia coli*

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ABSTRACT

The ribosome is a highly dynamic ribonucleoprotein machine. During assembly and during translation the ribosomal RNAs must routinely be prevented from falling into kinetic folding traps. Stable occupation of these trapped states may be prevented by proteins with RNA chaperone activity. Here, ribosomal proteins from the large (50S) ribosome subunit of *Escherichia coli* were tested for RNA chaperone activity in an in vitro *trans* splicing assay. Nearly a third of the 34 large ribosomal subunit proteins displayed RNA chaperone activity. We discuss a possible role of this function during ribosome assembly and during translation.

Keywords: RNA chaperone; ribosomal proteins; *trans* splicing; RNA folding; 50S subunit

INTRODUCTION

The eubacterial ribosome is a complex macromolecular machine composed of a small (30S) subunit including 16S rRNA and more than 20 proteins and a large (50S) subunit consisting of 23S and 5S rRNAs and more than 30 proteins. Understanding how this multitude of RNA and protein components assembles and functions is of considerable biological interest. First, the interdigitation of rRNA and protein components on the ribosome immediately suggests that assembly must be a complex process in which the ribosomal proteins play a critical role. Moreover, while much attention has recently focused on the role of the rRNA in the function of the ribosome, a glance at its structure reveals the extent to which the overall structure, and thus its function, must depend on the ribosomal proteins (Ban et al. 2000; Wimberly et al. 2000; Harms et al. 2001). Many of the ribosomal proteins have unusual extended basic tails that appear to wind their way through ribosomal RNA, acting to cement together the final structure. Most ribosomal proteins also have a globular domain that extends over the surface of the subunit, most often in solvent-exposed regions. There are, however, notable exceptions where the ribosomal proteins are located in the interface region between the large and small subunits of the ribosome where they might play direct roles in functionally critical intersubunit interactions like S12 and S13 (Culver et al. 1999; Cukras et al. 2003). In addition, while ribosomal proteins are largely absent from the peptidyl transfer and decoding regions, they are the principal components of the stalk region of the large ribosomal subunit that is directly involved in binding and interacting with translation factors like EF-Tu and EF-G (Mohr et al. 2002). Finally, a variety of ribosomal proteins are directly implicated in ribosome function based on documentation of their motions during discrete steps of translation (Gao et al. 2003; Valle et al. 2003).

We focus here on the potential role of ribosomal proteins as RNA chaperones to prevent the ribosome or other RNA molecules from stably occupying misfolded conformational states. Earlier work from Belfort and coworkers demonstrated that the small subunit ribosomal protein S12 from *Escherichia coli* has ATP-independent RNA annealing and RNA displacement activities in vitro (Coetzee et al. 1994). These activities are associated with RNA chaperones because their actions increase the fraction of correctly folded RNA by either impeding the formation of misfolded structures or by destabilizing folding intermediates (Herschlag 1995). In a separate work the relevance of the in vitro activity of ribosomal protein S12 could be extended to an in vivo context where S12 overexpression rescued a misfolded group I intron to promote catalysis (Clodi et al. 1999). In vivo it was further demonstrated that the RNA chaperone StpA destabilizes tertiary structural elements of a group I intron, again ultimately leading to correct folding and associated catalysis (Waldsich et al. 2002). While it is unclear

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whether the documented RNA chaperone activity of S12 is implemented by the ribosome for optimal function, it is clear that such activities might be useful to guarantee the dynamic flexibility of the ribosome during the translation cycle.

To extend the initial observation made with S12, we here focus on the purification of the ribosomal proteins from the large ribosomal subunit from *E. coli* and their evaluation in an in vitro RNA chaperone assay (Coetzee et al. 1994; Zhang et al. 1995). We found that nearly a third of the large ribosomal subunit proteins from *E. coli* display RNA chaperone activity.

RESULTS AND DISCUSSION

The large ribosomal subunit proteins were cloned, overexpressed, and purified similarily as previously described for the small ribosomal subunit proteins (Culver and Noller 1999). The overexpressed proteins were purified under denaturing conditions (6 M urea) over a cation column (with the exception of ribosomal protein L4, which was purified over an anion column) and eluted with an increasing salt gradient (see Table 1 and Materials and Methods). Purification was repeated when significant RNase activity remained. In the case that a second purification was performed, the respective second buffer, in which the proteins were dialyzed prior to the second purification, is indicated in Table 1. In some cases the same buffer as used for the first purification was chosen; in other cases the pH of the buffer was slightly increased to assure higher purity of the eluted proteins. We next used the previously developed *trans* splicing assay to evaluate the RNA chaperone activity of the collection of purified ribosomal proteins. In this assay the pre-mRNA of the *thymidylate synthase* (*td*) gene containing

TABLE 1. Purification of ribosomal proteins

Ribosomal protein	Solubility	FPLC column[a]	Buffer[b]	Elution (mM KCl)	pI[c]	Mw[d]
L1	insoluble	Resource S	A, B	270, 210	9.2	24.599
L2	insoluble	Resource S	B, B	280	>12.0	29.730
L3	insoluble	Resource S	C, C	175	9.7	22.258
L4	insoluble	Resource Q	C, D	180, 55	7.6	22.087
L5	insoluble	Resource S	B, A	flowthrough	9.4	20.171
L6	purified by Julie Brunelle	(unpubl.)			10.0	18.832
L7/L12	purified by Julie Brunelle	(unpubl.)			4.8	~12.2 each
L9	insoluble	Resource S	B, C	flowthrough	6.4	15.696
L10	purified by Julie Brunelle	(unpubl.)			7.5	17.581
L11	purified by Julie Brunelle	(unpubl.)			9.7	14.874
L13	insoluble	Resource S	A, B	255, 215	10.1	16.019
L14	purified by Julie Brunelle	(unpubl.)			12.3	13.341
L15	soluble	Resource S	B	260	12.0	14.981
L16	insoluble	Resource S	B, B	320	12.0	15.296
L17	soluble	Resource S	B, D	205	11.0	14.365
L18	insoluble	Resource S	B, D	280, 215	12.0	12.770
L19	insoluble	Resource S	B, B	180	>12.0	13.002
L20	soluble	Resource S	B, B	360	>12.0	13.366
L21	insoluble	Resource S	C	800	8.2	11.565
L22	soluble	Resource S	A, A	160	11.5	12.227
L23	soluble	Resource S	C, A	535, 200	9.6	12.209
L24	soluble	Resource S	A, A	155	10.7	11.185
L25	insoluble	Resource S	C	345	9.4	10.694
L27	soluble	Resource S	B, B	220	>12.0	8993
L28	insoluble	Resource S	B, A	320, 365	*11.42*	8875
L29	insoluble	Resource S	A, B	90, 60	10.0	7274
L30	soluble	Resource S	B, D	200	>12.0	6411
L31	soluble	Resource S	A, B	230, 160	*9.46*	6971
L32	soluble	Resource S	B, B	210	11.3	6315
L33	insoluble	Resource S	B, B	225	>12.0	6255
L34	soluble	Resource S	B	385	*>12.0*	*5381*

[a](Resource S) cation column; (resource Q) anion column.
[b]Buffer used for the FPLC column: For elution a gradient with increasing KCl concentrations was used. The salt concentration at which the protein was eluted is indicated (Elution mM KCl). Proteins with high impurity were purified a second time with the indicated buffer gradient. A: 20 mM Tris (pH 7.0 at 4°C), 6 M urea, 20 mM–1000 mM KCl (gradient). B: 20 mM Tris (pH 8.0 at 4°C), 6 M urea, 20 mM–1000 mM KCl (gradient). C: 20 mM NaAc (pH 5.6), 6 M urea, 20 mM–1000 mM KCl (gradient). D: 20 mM Tris (pH 9.5 at 4°C), 6 M urea, 20 mM–1000 mM KCl (gradient). Ribosomal proteins were purified up to two times each depending on their purity.
[c]pI from Kaltschmidt (1971); pIs printed in italic were calculated from http://us.expasy.org/tools/pi_tool.html.
[d]Molecular weights from Giri et al. (1984).

a group I intron is split in two halves. The first transcript, H1, contains the 5′ exon sequences (549 nt) and 131 nt of the intron and the second transcript, H2, contains the 3′ part of the intron (147 nt) and parts of exon 2 (23 nt) (Fig. 1A). The two independent constructs were transcribed using ^{35}S-α-UTP and were then incubated together. The group I intron *trans*-splicing reaction was initiated with the addition of ^{32}P-GTP, so that the resulting spliced product is doubly labeled (internally with ^{35}S-α-UTP and at the 5′ end with ^{32}P-GTP). *Trans* splicing was performed in the absence and the presence of the respective ribosomal protein. At 55°C the H1 and H2 RNAs effectively anneal and fold into a productive molecule while at 37°C the RNAs fail to assemble into an active conformation. The group I splicing reaction is sensitive to lower temperatures. The individual purified ribosomal proteins were added to a final concentration of 2 μM as was used recently for assessing the RNA chaperone activity of StpA (Mayer et al. 2002). Higher protein concentrations (3 μM, 4 μM, 6 μM) were tested for ribosomal protein L19 and did not show an increase in the observed chaperoning activity. Addition of an RNA chaperone at 55°C does not further stimulate *trans* splicing (data not shown). Only when *trans* splicing was already reduced due to lower temperatures (37°C) does the presence of an RNA chaperone stimulate *trans* splicing. Figure 1B shows a representative time course of *trans* splicing in the presence of ribosomal protein L19 where the reaction is five times more efficient with L19 at 37°C than without L19 at 55°C (positive control). Ribosomal proteins L1, L13, and L15, among others, further increased *trans* splicing at 37°C, whereas others (L9, L7/L12) did not stimulate *trans* splicing (Fig. 2). Figure 3 summarizes the relative *trans* splicing rates of proteins from the large ribosomal subunit where proteins L1, L3, L13, L15, L16, L18, L19, L22, and L24 significantly stimulated *trans* splicing. More moderate stimulation of *trans* splicing was observed with L4 and L17. Next we asked if a combination of two proteins further increases the splicing rates. Ribosomal proteins L4 and L24 both assemble to a short fragment within 23S rRNA (Stelzl and Nierhaus 2001) suggesting that together they might act cooperatively. However, addition of L4 together with L24 did not further stimulate *trans* splicing (data not shown).

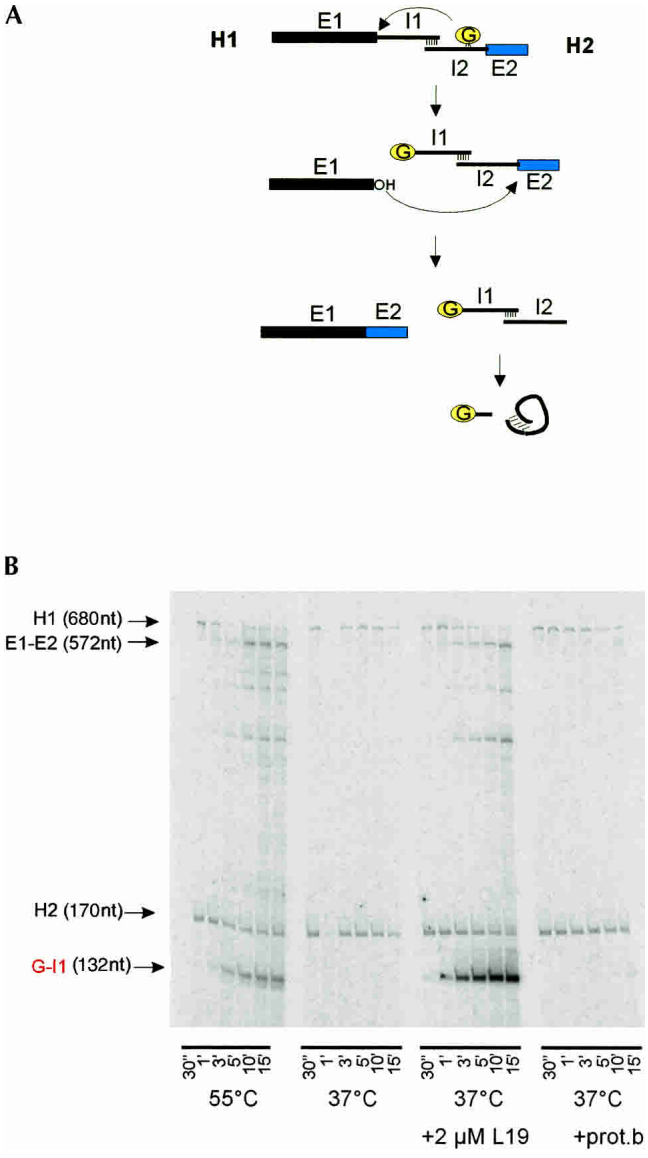

FIGURE 1. Stimulation of *trans* splicing by ribosomal protein L19. (*A*) Scheme of the *trans* splicing assay: RNAs H1 and H2 are annealed and splicing is started by the addition of the guanosine cofactor. (E1) exon 1, (I1) 5′ part of the intron, (I2) 3′ part of the intron, (E2) exon 2. (*B*) *Trans* splicing polyacrylamide gel. The substrates H1, H2, and the product (guanosine-5′-intron) and the ligated exons are indicated. The first set (lanes *1–6*) shows a time course of *trans* splicing in the absence of proteins at 55°C and the following set shows splicing at 37°C. The third set shows *trans* splicing at 37°C in the presence of 2 μM L19. The final set shows *trans* splicing in the presence of ribosomal protein storage buffer.

Ribosomal proteins L1 and L19 showed the highest levels of RNA chaperone activity in our assay. (L1: relative splicing rate four times over positive control, L19: relative splicing rate five times over positive control). Ribosomal protein L19 is located at the subunit interface and interacts with L14, L3, and with rRNA elements of both subunits (Harms et al. 2001). Ribosomal protein L1 consists of two globular domains and is localized to the stalk region near the E-site. Previous studies indicate that this is a highly flexible region of the ribosome (Stark et al. 2000; Uchiumi et al. 2002). It has been further suggested that L1 plays a role in facilitating E-site tRNA release, an activity that may be facilitated by RNA chaperone activity (Agrawal et al. 1999; Gomez-Lorenzo et al. 2000; Harms et al. 2001; Yusupov et al. 2001). Finally, L1 is a translational repressor that regulates expression of its own mRNA in *E. coli* (Nomura et al. 1984; Nikonov et al. 1996). While general models for translation control by several ribosomal proteins are based on sequestration of the Shine–Dalgarno region, it is possible that these proteins directly resolve RNA structures to promote

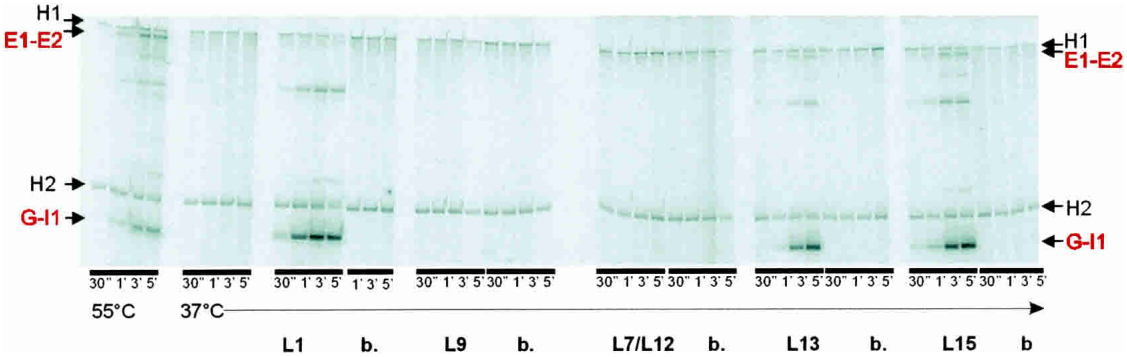

FIGURE 2. *Trans* splicing at 37°C in the presence of ribosomal proteins L1, L9, L7/L12, L13, and L15. The polyacrylamide gel shows a time course of *trans* splicing in the absence of ribosomal proteins at 55°C (first set of lanes *1–4*) and at 37°C (second set of lanes *5–8*). It follows the time courses in the presence of 2 μM ribosomal proteins L1, L9, L7/L12, L13, and L15. Lanes indicated with "b" correspond to sets of *trans* splicing performed in the presence of ribosomal protein storage buffer, where the same volume as for each ribosomal protein was used.

their own repressor activities. A similar model has recently been proposed for another RNA chaperone, Hfq, which binds to and resolves RNA structural elements (Geissmann and Touati 2004). Ribosomal protein L3 is one of the early assembling proteins (Rohl and Nierhaus 1982) and is composed of a globular domain and an unstructured extension (Ban et al. 2000; Harms et al. 2001). The unstructured extension has a methylated glutamine residue and the loss of this modification results in cold-sensitive ribosome assembly (Chang and Chang 1975; Lhoest and Colson 1981). Perhaps L3 directly facilitates RNA rearrangements in this region of the ribosome that are critical in preventing the long-term sampling of cold-sensitive metastable states. Finally, like L1, ribosomal protein L4 is a negative transcriptional and translational regulator of its own operon (Lindahl and Zengel 1979; Yates et al. 1980; Zengel et al. 1980), consisting of a globular domain with a long unstructured extension, like L3 (Ban et al. 2000; Harms et al. 2001). Both L4 and L22 are located along the exit tunnel of the ribosome, suggesting potential roles in regulating the extrusion of the growing peptide (Nissen et al. 2000; Gabashvili et al. 2001).

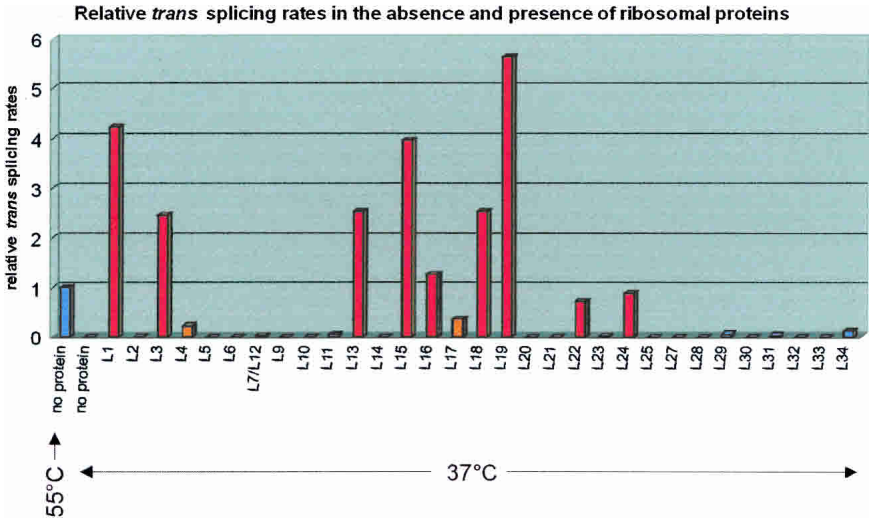

FIGURE 3. Summary of *trans* splicing rates in the absence and in the presence of ribosomal proteins L1 to L34 from *E. coli*. The first two bars labeled 55° and 37° show relative *trans* splicing rates at 55°C and 37°C in the absence of proteins. The following bars show relative rates of *trans* splicing in the presence of the indicated ribosomal protein at 2 μM final concentration all measured at 37°C. Bars in red indicate that these ribosomal proteins display strong RNA chaperone activities. Ribosomal proteins L4 and L17 (in orange) show intermediate stimulation of *trans* splicing. Relative splicing rates are obtained from one to five individual experiments and calculated by the formula $(nx - n37)/(n55 - n37)$ for each polyacrylamide gel separately, where n is the relative splicing rate of either x (the respective ribosomal protein) or 55 (at 55°C) or 37 (at 37°C, the latter two in the absence of a protein).

Most of the ribosomal proteins are located at the periphery of the ribosome and are thought to play critical roles both in assembly and stabilization of the ribosome structure. Some of the ribosomal proteins that have significant RNA chaperone activity in our assay assemble at early stages of reconstitution (e.g., L3 and L4) (Nierhaus 1991). Other proteins that we have identified are involved at later stages during assembly (e.g., L15, L16) (Franceschi and Nierhaus 1990). It is easy to imagine that RNA chaperone activity could be important at all stages of assembly, ensuring that the RNA does not become trapped in nonnative structures. It is also possible that the ribosomal proteins play critical roles in facilitating RNA rearrangements during translation, and that these movements depend on the RNA chaperone-like properties of these unusual proteins. We propose that the RNA chaperone activity of the ribosomal proteins identified here, besides preventing the formation of misfolded structures during assembly, could be

more generally critical to the dynamic rearrangements of the ribosome during tRNA selection, peptide bond formation, and translocation.

MATERIALS AND METHODS

Cloning, expression, and purification of ribosomal proteins

Genomic DNA from *E. coli* MRE600 was used for PCR amplification. The PCR primers were chosen to have NdeI (5′) and BamHI (3′) sites except for ribosomal protein L16, where primers with NdeI and EcoRI sites were used. The PCR product was cloned into the vector pET24b (Novagen). The plasmid for L1 purification was provided by Hyuk Kyu Seoh, the plasmids for L10, L11, and L12 were provided by Lovisa Holmberg, and the plasmids containing L6 and L14 were provided by Julie Brunelle. Plasmids L2, L3, L4, and L15 were provided by Kate Liebermann. The ribosomal proteins were overexpressed in *E. coli* strain BLR1. After 4 h of induction the cells were collected, resuspended in 20 mM Tris (pH 7.0) at 4°C, 1 M KCl, 6 mM β-mercaptoEtOH, and cracked with a French Press. The lysate was centrifuged at 4°C to either clear the supernatant or pellet the insoluble protein. The pellet was solubilized in the respective buffer (see Table 1). The resuspended pellet or supernatant was dialyzed at 4°C overnight into the respective FPLC buffer (Table 1). The lysate was then purified over a resource S cation column (or a resource Q anion column). The proteins were eluted over a salt gradient (20 mM KCl–1000 mM KCl) and dialyzed into storage buffer (20 mM Tris at pH 7.4, 4 mM $MgAc_2$, 400 mM NH_4Cl, 0.2 mM EDTA, 5 mM β-mercaptoEtOH) except for L4, which was stored in buffer C (Table 1) and dialyzed into storage buffer prior to usage. The purity of the ribosomal proteins was tested on Coomassie-stained SDS urea gels. The ribosomal proteins were further evaluated for RNase activity by incubating the ribosomal protein with transcribed RNA and testing RNA degradation by separation on a 5% polyacrylamide gel (PAGE). Ribosomal proteins that still showed a significant amount of RNase activity were again purified either at the same pH or at a slightly increased pH and were eluted with the increasing salt gradient (20 mM KCl–1000 mM KCl). Then the proteins were dialyzed into the storage buffer (20 mM Tris at pH 7.4, 4 mM $MgAc_2$, 400 mM NH_4Cl, 0.2 mM EDTA, 5 mM β-mercaptoEtOH).

In vitro transcription

The plasmids for H1 (exon 1 + 5′ intron) and H2 (3′ intron + exon 2) were linearized with SalI (for H1) and BamHI (for H2). Construct H1 consists of 549 nt of exon 1 and 131 nt of the 5′ part of the intron (total 680 nt). Construct H2 consists of 147 nt of the 3′ half of the intron and 23 nt of exon 2 (total 170 nt). The RNAs H1 and H2 were transcribed with 40 mM Tris (pH 7.0), 26 mM $MgCl_2$, 3 mM spermidine, 5 mM ATP, 5 mM GTP, 5 mM CTP, 2.5 mM UTP, 2.5 mM ^{35}S-α-UTP, 10 mM DTT, T7 RNA polymerase at 37°C for 3 h, followed by 30 min DNase digest and purification of the transcripts over a 5% polyacrylamide gel (PAGE).

Trans splicing assay

We incubated 200 nM H1 and 200 nM H2 transcripts 1 min at 95°C and cooled them to either 55°C (for the positive control) or 37°C. Next, splicing buffer (4 mM Tris at pH 7.4, 3 mM $MgCl_2$, 0.4 mM spermidine, 4 mM DTT final concentration) and 0.33 pmol ^{32}P-GTP were added. Additionally, either the respective ribosomal protein was added to a final concentration of 2 μM or the same quantity of ribosomal storage buffer (20 mM Tris at pH 7.4, 4 mM $MgAc_2$, 400 mM NH_4Cl, 0.2 mM EDTA, 5 mM β-mercaptoEtOH) was added. The reactions were incubated at either 55°C (positive control) or at 37°C and aliquots were stopped by adding a final concentration of 40 mM EDTA and 300 μg/mL tRNA. The samples were phenol-$CHCl_3$ extracted, precipitated, and loaded on 5% polyacrylamide gels. Bands corresponding to the product of the first step of splicing (intron 1 with 5′ added guanosine) were measured by PhosphorImager and relative rates were calculated for each gel by setting the positive control to 1 (*trans* splicing at 55°C in the absence of ribosomal proteins) and subtracting the negative control (*trans* splicing at 37°C in the absence of ribosomal proteins) from each obtained rate on each gel by the formula: $(nx - n37)/(n55 - n37)$, nx being the relative splicing rate in the presence of the respective ribosomal protein, $n55$ being the relative splicing rate at 55°C in the absence of ribosomal proteins, and $n37$ being the relative splicing rate at 37°C in the absence of a ribosomal protein.

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