Protein factors associated with the SsrA·SmpB tagging and ribosome rescue complex

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SsrA RNA acts as a tRNA and mRNA to modify proteins whose synthesis on ribosomes has stalled. Such proteins are marked for degradation by addition of peptide tags to their C termini in a reaction mediated by SsrA RNA and SmpB, a specific SsrA-RNA binding protein. Evidence is presented here for the existence of a larger ribonucleoprotein complex that contains ribosomal protein S1, phosphoribosyl pyrophosphate synthase, RNase R, and YfbG in addition to SsrA RNA and SmpB. Biochemical, genetic, and phylogenetic results suggest potential roles for some of these factors in various stages of the ribosome rescue and tagging process and/or the presence of functional interactions between one or more of these proteins and SsrA.

tmRNA | 10Sa RNA | trans translation | protein-RNA recognition | VacB

In bacteria, SsrA RNA (also known as tmRNA or 10Sa RNA) acts both as a tRNA and an mRNA in a process that clears stalled ribosomes and tags the nascent polypeptides associated with such ribosomes with a C-terminal peptide that results in their degradation (1–3). The 5′ and 3′ ends of SsrA RNA form an alanyl-tRNA-like domain with a disrupted stem replacing the normal tRNA anticodon stem–loop (4, 5). Connecting the 5′ and 3′ portions of this SsrA structure are pseudoknot 1 (PK1), the degradation tag-reading frame, and pseudoknots 2, 3, and 4 (PK2, PK3, and PK4) (Fig. 1). SmpB protein is required for the biological activities of SsrA, binds specifically to SsrA RNA, and is necessary for the association of SsrA with ribosomes *in vivo* (6). Strains lacking SmpB have the same phenotypes as SsrA-defective strains. The SsrA·SmpB system is conserved in all bacteria, suggesting that the ribosome-rescue and protein quality-control functions are important for bacterial survival (3, 7).

Three additional proteins, all part of the general translation machinery, have been shown to interact with SsrA RNA. SsrA RNA is charged with alanine by alanyl-tRNA synthetase (1), and aminoacylated SsrA forms a ternary complex with elongation factor (EF)-Tu and GTP (8, 9). Complex formation with EF-Tu·GTP protects the labile ester linkage of charged SsrA (9) and, by analogy with charged tRNAs, undoubtedly is important for delivery of alanyl-SsrA to ribosomes. Recent studies have shown also that ribosomal protein S1 binds SsrA RNA and is required for its ribosome association *in vitro* (10). Here, we describe the identification of PrsA, RNase R (VacB), YfbG, and ribosomal protein S1 as protein factors that copurify with SsrA and SmpB. Potential roles for these proteins and evidence for functional interactions between these proteins and SsrA RNA are discussed.

Materials and Methods

Microbiology. All strains were derivatives of *Escherichia coli* K12 and were grown in LB medium (10 g tryptone/5 g yeast extract/5 g NaCl per liter) or on LB-agar plates containing ampicillin (100 μg/ml), kanamycin (50 μg/ml), chloramphenicol (30 μg/ml), or tetracycline (30 μg/ml) as needed. Strains W3110 *rnr*::*cat* and W3110 *prsA*::*kan* were constructed by P1 transduction of the *rnr*::*cat* gene disruption from strain CA265 *rnr*::*cat* (11) and the *prsA*::*kan* gene disruption from strain HO773 (12), respectively. Strains W3110 *ssrA*::*kan* and W3110 Δ*smpB-1* have been described previously (6). The plasmid encoding SsrA-H_6 (pKW24) was a gift from Eric Roche (MIT, Cambridge, MA; ref. 3). Plasmids expressing SsrA pseudoknot variants pK1L, pK2L, pK3 l, and pK4L were a gift of Akira Muto (Hirosaki University, Hirosaki, Japan; ref. 14). The tandem *smpB* and *ssrA* genes of *E. coli* were amplified by PCR to add an *Nde*I site that overlaps the ATG initiation codon at the 5′ end of the *smpB* coding sequence and a *Bam*HI site just past the 3′ end of the *ssrA* gene. This DNA fragment was cut with both enzymes and cloned into *Nde*I- and *Bam*HI-digested pET28b (Novagen) to generate pETBA. The coding sequences of the *E. coli* genes for ribosomal protein S1, *prsA*, *rnr*, and *yfbG* were amplified by PCR and cloned into pET28b as described above. Plasmids were transformed into *E. coli* strain BL21(DE3)/pLysS for protein expression. *E. coli* cells deleted for *prsA* can grow in rich media containing NAD, albeit at much reduced rates (12). This growth defect was complemented by low-copy-number plasmids expressing His_6–PrsA.

Testing for SsrA-tagging of the model substrate λ-*N*-trpAt (2), determining the plating efficiency of phage λ*imm*P22 *dis c*2–5, and measuring induction of phage Mu *c-ts62 pAp1* in lysogens were performed as described (6) in pETBA transformants of strains W3110 Δ*smpB-1* and W3110 *ssrA*::*kan*.

Complex Purification. *E. coli* strain BL21(DE3)/pLysS/pETBA was grown in 6 liters of LB broth at 37°C to an A_{600} of 0.6, induced with 1 mM isopropyl β-D-thiogalactoside, and grown for an additional 0.5–3 h. Cells were harvested, resuspended in 150 ml of buffer A (50 mM KPi, pH 6.5/250 mM KCl/1 mM β-mercaptoethanol/2 mM $MgCl_2$/15 mM imidazole), and lysed by sonication. Cellular debris was removed by centrifugation at 15,000 rpm in a Sorvall SS-34 rotor, and the supernatant was loaded on a 1-ml Ni^{2+}-nitrilotriacetic acid (NTA) column (Qiagen, Chatsworth, CA). The column was washed with 20 volumes of buffer A, and bound proteins were eluted with 200 mM imidazole in buffer A. Fractions containing SmpB protein were combined and loaded directly onto a HiPrep 16/60 Sephacryl S300 FPLC column (Amersham Pharmacia) equilibrated in buffer B (same as buffer A but with 200 mM KCl and no imidazole). Column fractions were analyzed by SDS/PAGE, pooled accordingly, and stored frozen at −80°C.

Identification of SsrA·SmpB-Associated Proteins. For N-terminal sequencing, protein samples were electrophoresed on 12% Laemmli SDS gels, transferred to a poly(vinylidene difluoride) (PVDF) membrane, strained with Coomassie brilliant blue R250, and washed with water. Individual bands were excised and subjected to sequential Edman degradation by the MIT Biopolymers Facility (Cambridge, MA). For mass spectrometry, individual bands were excised from silver-stained gels and digested

Abbreviations: NTA, nitrilotriacetic acid; PrsA, phosphoribosyl pyrophosphate synthase; PVDF, poly(vinylidene difluoride).

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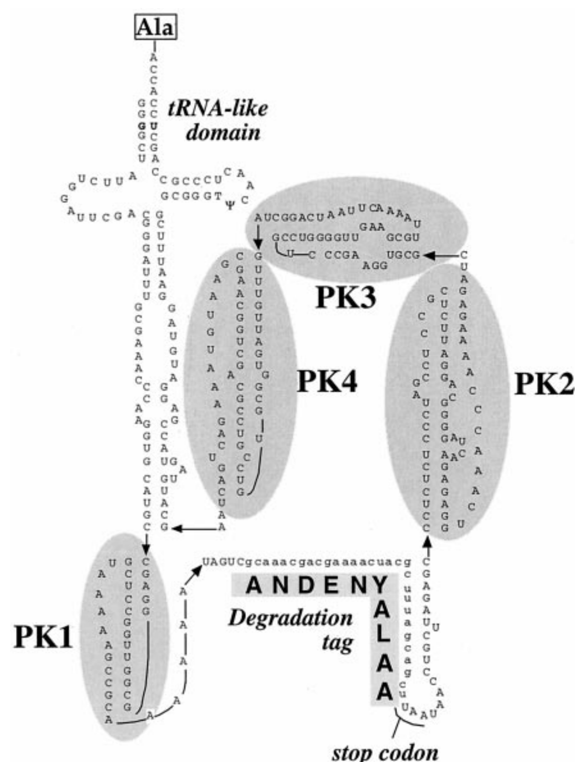

Fig. 1. Predicted secondary structure of SsrA RNA (4). The tRNA-like domain, pseudoknots, and degradation tag-coding sequence are marked.

with 80 ng of modified trypsin (Roche Diagnostics) in 1 μl. Tryptic peptides were eluted from gel slices by extracting with 5% formic acid and 50% acetonitrile and dried under a vacuum. After resuspension in 0.1% trifluoroacetic acid, peptides were bound to C18 Zip tips (Millipore) and eluted by using a 50% acetonitrile solution containing α-cyano-4-hydroxy-cinnamic acid. This material was spotted directly onto target plates for matrix-assisted laser desorption ionization/time-of-flight mass spectrometry by using a PerSeptive Biosystems (Framingham, MA) Voyager instrument. Only peaks significantly above background, which was determined by processing an empty portion of the gel, were selected. Searches of the SwissProt database of *E. coli* protein sequences were performed by using MS-FIT (http://prospector.ucsf.edu).

Northern and Western Blots. RNA from purified complex was electrophoresed on a 1.5%-agarose 20%-formaldehyde gel and transferred in 20× SSC to a Nytron membrane (Schleicher and Schuell). Prehybridization and hybridization with appropriate ^{32}P-labeled DNA oligonucleotides complementary to SsrA RNA (5′-TAA AGC GTA GTT TTC GTC GTT TGC-3′) or 16S ribosomal RNA (5′-CCG TCC GCC ACT CGT CAG CAA-3′) were performed according to standard protocols. For Western blot analysis, purified complex or total cellular proteins were electrophoresed on 10–15% Laemmli SDS gels and transferred to PVDF membranes. These blots were probed with antibodies specific to each protein.

Gel-Mobility Shift Assays. A form of SsrA corresponding to mature processed RNA and four pseudoknot variants were transcribed *in vitro* by using T7 polymerase in the presence of [α-^{32}P]UTP. Binding mixtures (20 μl) contained 50 mM Tris (pH 7.5), 100 mM KCl, 5% glycerol, 1 mM DTT, 0.01% Nonidet P-40, 0.1 mg/ml BSA, 100 pM of labeled SsrA RNA, and different amounts of each protein. Mixtures were incubated at room temperature for 30 min and loaded onto an 8% polyacrylamide gel in TBMg buffer (50 mM Tris, pH 8.5/25 mM borate/0.1 mM $MgCl_2$). The gel was electrophoresed at 200 V, dried on gel-drying paper, and exposed to BIOMAX MR film (Eastman Kodak) for 8–12 h at −80°C. Competition experiments were performed in the same manner except for inclusion of SsrA or yeast tRNA (Sigma) in the reaction mixtures.

Analysis of Endogenous SsrA–H_6 Tagging. For these experiments, strains were transformed with pKW24, which encodes SsrA-H_6, a mutant that adds a His_6 epitope as part of the mutant SsrA tag (13). Cultures of 1 liter were grown to an A_{600} of 0.8–0.9, harvested, resuspended in 20 ml lysis buffer (8 M urea/1% triton/5 mM β-mercaptoethanol/100 mM NaH_2PO_4/10 mM Tris, pH 8), and lysed by stirring for 1 h. Cell debris was removed by centrifugation for 30 min at >30,000 × *g*, and the supernatant was mixed with 1 ml of a Ni^{2+}-NTA slurry equilibrated in lysis buffer and allowed to bind with rocking for 1 h. The resin was loaded into a column and washed twice with 20 ml of lysis buffer. Proteins were eluted with 2 ml of elution buffer (8 M urea/1% triton/1 mM β-mercaptoethanol/0.1 M acetic acid), and the pH was raised by adding 1:10 (vol/vol) of 2 M Tris (pH 9.5). A portion of this mixture (80–100 μl) was resolved by electrophoresis on a 12% Laemmli SDS gel. Western blots of these samples were developed with His probe H-15 HRP antibodies (Santa Cruz Biotechnology).

Results

Vectors with Improved His_6–SmpB Solubility. We sought to use His_6–SmpB as bait to determine whether additional cellular factors associate with the SsrA·SmpB complex. Previous experiments, however, showed that overexpression of SmpB or His_6–SmpB resulted in formation of inclusion bodies that needed to be refolded *in vitro* for functional studies (6). Because SmpB and SsrA RNA form a stable complex *in vitro*, we reasoned that SmpB folding *in vivo* might require the presence of higher levels of SsrA RNA and that co-overexpression of SmpB and SsrA might result in production of a soluble complex. Toward this end, we cloned the tandem *smpB* and *ssrA* genes of *E. coli* under control of a T7 promoter in the pET28b expression vector to generate a plasmid called pETBA. In this plasmid, 20 amino acids including a His_6-tag were added to the N terminus of the SmpB protein. In genetic studies, pETBA complemented the phenotypes of strains lacking SmpB protein or SsrA RNA, showing that it produces biologically active SmpB and SsrA. Specifically, *ssrA*$^-$ or *smpB*$^-$ strains transformed with pETBA-plated phage λ*imm*P22 *dis c*2–5 as well as wild-type strains, showed wild-type induction of temperature-sensitive Mu lysogens, and showed wild-type levels of SsrA tagging of model substrates (6).

SsrA·SmpB Complex and Associated Factors. Following purification of His_6–SmpB protein from strain BL21(DE3)/pLysS/pETBA by Ni^{2+}-NTA chromatography and gel filtration (Fig. 2*A*), we found that the protein copurified with RNA, as judged by strong absorbance at 260 nm, resistance to DNase treatment, and sensitivity to RNase and hydroxide treatment. Northern blot analysis demonstrated hybridization with a probe specific for SsrA RNA but not with a probe for 16S ribosomal RNA. Hence, SmpB protein and SsrA RNA purify as a complex.

SDS/PAGE and silver staining of the Ni^{2+}-NTA-purified material revealed a band corresponding to His_6–SmpB (≈21 kDa) and numerous additional polypeptides (Fig. 2*B*). After gel-filtration chromatography on Sephacryl S300 in 200 mM KCl (Fig. 2*A*), most of these additional polypeptides eluted in the same fractions as SmpB and SsrA (Fig. 2*B*), suggesting that they belong to a single ribonucleoprotein complex. On the basis of the elution positions of size markers from the same resin, the size of

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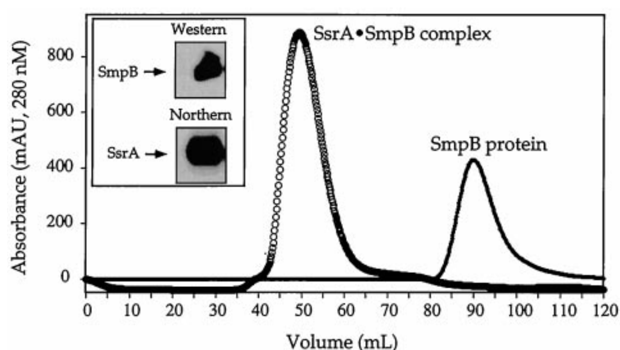

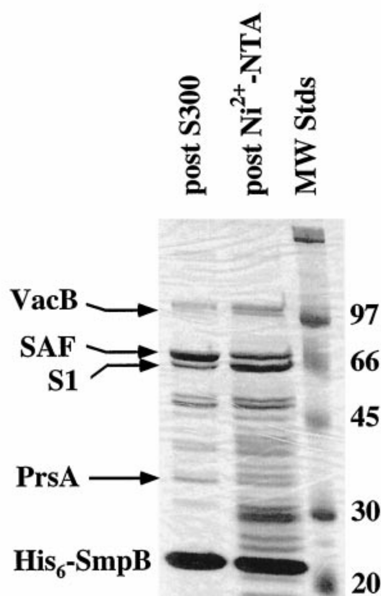

Fig. 2. Purification of SsrA·SmpB associated factors. (*A*) Elution of complex from Sephacryl S300 gel-filtration column. The elution profile of SmpB protein alone is shown for comparison. (*Inset*) Western and Northern blot analysis of the main peak for the presence SmpB protein and SsrA RNA, respectively. (*B*) SDS/PAGE analysis following Ni^{2+}-NTA and Sephacryl S300 columns. After each purification step, samples were concentrated 10-fold and electrophoresed on a 12% Laemmli SDS gel. Arrows mark the positions of identified polypeptides. Molecular mass markers are shown on right.

this complex was estimated to be in excess of 450 kDa. The most prominent of the additional polypeptides in the post-S300 complex had apparent molecular masses of approximately 97, 75, 65, 50, 40, and 35 kDa.

To identify the associated polypeptides, the pooled complex was electrophoresed on preparative SDS gels after gel filtration, and individual bands were excised and characterized by N-terminal Edman degradation and matrix-assisted laser desorption ionization/time-of-flight mass spectrometric analysis of tryptic peptides (Table 1). Data from these experiments were used in database searches of the *E. coli* genome to determine the identity of each factor. The p65 polypeptide was identified as ribosomal protein S1; consistent with this identification, anti-S1 antibodies crossreacted with p65 in western blots. The p35 polypeptide was identified as phosphoribosyl pyrophosphate synthase (PrsA). The p75 polypeptide corresponded to a protein of unknown function encoded by the *yfbG* gene. Homology searches using PSIBLAST (15) suggested that this protein consisted of two structural domains, the first homologous to methionine tRNA formyl transferase and the second homologous to sugar dehydratases and epimerases. We refer to the p75

Table 1. Factors identified in complex

Complex components	Matched tryptic peptides	N-terminal sequence	Western/Northern
His_6-SmpB	7/11	NH_2-GSSHHHH	+++
S1	10/12	NH_2-TESFAQL	+++
PrsA	9/13	NH_2-PDMKLF	antiserum unavailable
SAF	11/15	NH_2-MKTVVF	antiserum unavailable
RNase R (VacB)	8/10	none determined	antiserum unavailable
SsrA RNA	not applicable	not applicable	+++

polypeptide as SAF (for *S*srA-*A*ssociated *F*actor). Several additional bands with apparent masses near 97 kDa were analyzed also, although the intensities of these bands varied from one purification to another. Attempts to identify these polypeptides by N-terminal sequence analysis and peptide mass fingerprinting were unsuccessful initially. However, by concentrating samples and using gradient gels, one of these bands was excised cleanly and identified as RNase R (VacB) by peptide mass fingerprinting (Table 1). The identities of the remaining bands in the purified complex could not be determined unambiguously.

Factor Binding to SsrA RNA. To evaluate whether the SsrA·SmpB-associated factors bound directly to SsrA RNA, we attempted to clone, overexpress, and purify each factor. Cloning and/or solubility problems prevented successful purification of SAF and RNase R. His_6-tagged versions of S1 and PrsA were purified, however, and their binding to SsrA RNA and variants was analyzed by gel-mobility shift experiments.

Addition of increasing concentrations of S1 resulted in formation of an S1·SsrA–RNA complex (Fig. 3*A*). The observed binding was saturable with half-maximal binding at a free-S1 concentration of approximately 30 nM (Fig. 3*B*). To probe the specificity of S1·SsrA binding, unlabeled SsrA RNA and total-yeast tRNA were used to compete for binding of 100 nM S1 to 100 pM ^{32}P-labeled SsrA. Approximately 700-fold higher molar concentrations of yeast tRNA than SsrA RNA were required to achieve the same degree of competition (data not shown). SsrA RNA is predicted to have four pseudoknots (4, 5; Fig. 1). Moreover, in SELEX experiments, S1 binds preferentially to pseudoknots (16). As shown in Fig. 3*C*, S1 binding to SsrA variant missing PK1 was unaffected, whereas binding to a variant missing PK3 was reduced at least 10-fold. Analysis of S1 binding to SsrA variants missing PK2 or PK4 showed binding reduced by approximately 4-fold. These data suggest that PK3 may form part of the binding site for S1 binding but also implicate other regions of SsrA in stabilizing S1 interactions. We note that it also is possible that more than one S1 protein binds to SsrA RNA.

His_6–PrsA also bound to SsrA RNA, but with half-maximal binding at a free-PrsA concentration of approximately 1–2 μM (Fig. 4). The specificity of this interaction was probed by competition of unlabeled SsrA RNA or total-yeast tRNA for binding of 800 nM PrsA to 100 pM ^{32}P-labeled SsrA. Approximately 4-fold higher molar concentrations of yeast tRNA than SsrA RNA were required to achieve the same degree of competition (data not shown). Because SsrA is approximately four times the size of tRNA, there is clearly almost no specificity in terms of the weight of the competitor. The binding of PrsA to SsrA RNA was improved only slightly (2- to 4-fold) by the presence of SmpB protein in the binding reaction. These results suggest that higher-affinity or more-specific binding of PrsA to SsrA RNA may require the presence of other protein factors, SsrA base modifications (17), or small molecules that interact with PrsA. Because of the apparent lack of specificity in

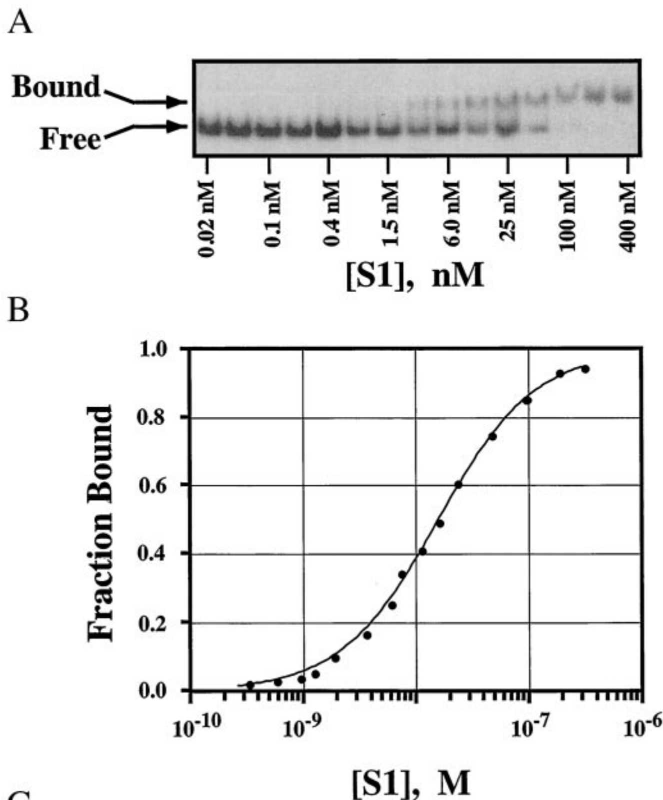

Fig. 3. (*A*) Gel-mobility shift assay of the binding of purified His_6-tagged ribosomal protein S1 to 100 pM of ^{32}P-labeled SsrA RNA produced by transcription *in vitro*. Arrows mark the positions of the bound and free RNA species. (*B*) Binding curve generated from data in *A*. (*C*) Binding of His_6-tagged ribosomal protein S1 to 100 pM ^{32}P-labeled PK1L and PK3L SsrA variants.

PrsA·SsrA interactions, binding to the pseudoknot variants was not attempted.

SsrA-Mediated Tagging in Mutant Strains. To test whether RNase R affects SsrA·SmpB-mediated tagging, we transformed a strain containing an interruption of the *rnr* gene (11) with a plasmid expressing SsrA-H_6. This SsrA variant encodes a tag sequence ending with a His_6 epitope that blocks rapid degradation and permits detection of endogenously tagged proteins by using anti-His_6 antibodies (13). When cellular proteins were separated by SDS/PAGE, transferred to PVDF membranes, and probed with anti-His_6 antibody, endogenous tagging was absent in cells lacking SmpB as expected but was detected readily in the *rnr*::*cm* cells (Fig. 5). Interestingly, when compared with an otherwise-isogenic strain, cells lacking functional RNase R consistently displayed higher levels and a somewhat different pattern of tagged proteins. These results show that RNase R is not required for tagging but indicate that it plays some role in the tagging process.

Normal tagging of endogenous proteins was observed in *prsA*-defective cells containing the SsrA–H_6 plasmid (data not shown), showing that PrsA is not required for SsrA·SmpB-mediated tagging. Strains defective in S1 or SAF were not available to test the effects of mutations in these genes on tagging.

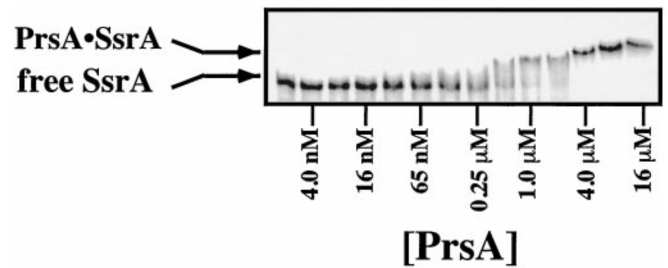

Fig. 4. Gel-mobility shift assay of the binding of purified His_6-tagged PrsA protein to 100 pM of ^{32}P-labeled SsrA RNA. Arrows mark the bound and free positions.

Discussion

Although the tmRNA model is reasonably well established (for review, see ref. 3), many questions remain. How does SsrA distinguish a stalled ribosome from a ribosome engaged in normal translation? How is the correct codon chosen for the first tag residue encoded by SsrA? What is the fate of the damaged mRNA replaced by SsrA? Do additional cellular factors participate in any of the tagging and ribosome-rescue activities? The studies reported here have identified four proteins that associate with SsrA·SmpB. This discovery raises two additional questions. Are these interactions biologically meaningful? Can we deduce plausible roles for some of these factors in the SsrA·SmpB system? Below, we discuss each of these four proteins.

Our finding that ribosomal protein S1 copurified with the SsrA·SmpB complex and bound strongly and specifically to SsrA RNA confirmed studies reported while this work was in progress (10). Wower *et al.* (10) detected direct and specific S1·SsrA

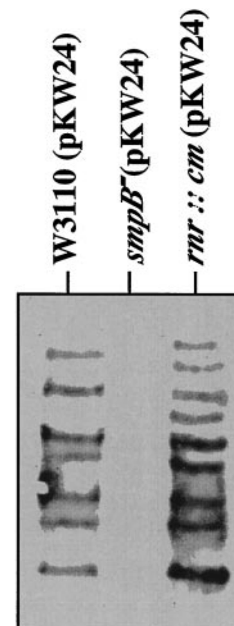

Fig. 5. Western-blot analysis of SsrA–H_6-tagged proteins in W3110/pKW24 cells, W3110 *smpB*Δ*-1*/pKW24 cells, and W3110 *rnr*::*cm*/pKW24 cells. Cell lysates from each strain were prepared as described in *Materials and Methods*, resolved by electrophoresis on a 12% SDS/PAGE, transferred to PVDF membrane, and probed with anti-His_6 antiserum.

binding and used crosslinking studies to define sites of contact between S1 and individual bases in pseudoknots PK2, PK3, and the PK3–PK4 junction, as well as a contact in the tag-coding region of SsrA. The majority of the nucleotides crosslinked to S1 were within PK3, in agreement with our finding that PK3 is an important determinant of S1 binding. When S1 was depleted from *in vitro*-translation extracts, SsrA RNA did not associate with ribosomes (10). S1 is conserved in most bacteria and has been implicated in the selection of translation-start sites, especially for mRNAs lacking Shine–Dalgarno ribosome binding sites upstream of the ATG initiation codon (16, 18). It seems likely, therefore, that binding of one or more S1 proteins plays some role in helping the SsrA·SmpB complex bind ribosomes and reinitiate transcription correctly on the peptide-reading frame. SsrA variants that individually replace pseudoknots 2, 3, and 4 still show tagging activity *in vitro* (14), but our studies show that S1 still binds to these variants, although with lower affinities. These results make it unlikely that S1 interactions with any of the individual pseudoknots in SsrA RNA are required for tagging, unless S1 functions mainly to disrupt the structure of these pseudoknots.

RNase R (VacB), a member of the RNase II family (11), consists of a 3′-to-5′ exonuclease domain and an S1-like RNA-binding domain. Because RNase R was a relatively minor component of the purified complex, we questioned the significance of this association. We were unable to purify RNase R for biochemical studies but note several observations that support a functional relationship between this exonuclease and SsrA·SmpB. First, we found that patterns of SsrA tagging were altered in a strain lacking active RNase R. Second, the *rnr* gene is located immediately upstream of the *smpB* gene in numerous bacteria, and prokaryotic genes in the same pathway are clustered frequently (19). The functional-coupling algorithm available on the IGWIT site (http://igweb.integratedgenomics.com/IGwit/) assigns a high degree of significance to this colocalization of *rnr* and *smpB*. Finally, *ssrA* mutations in *Salmonella typhimurium* result in up-regulation of three genes, one of which is *rnr* (20). What does RNase R do? Because the level of SsrA–H_6 tagging of endogenous proteins increases 2- to 4-fold in an *rnr* mutant, it seems likely that it plays some role in degradation of the mRNA that is replaced by SsrA RNA during tagging and ribosome rescue. The fact that the pattern of tagging also is altered may suggest that some mRNAs on stalled ribosomes are more susceptible to RNase R degradation than others.

PrsA is an enzyme required for *de novo* synthesis of nucleotides, tryptophan, and histidine (21–23). The growth phenotypes of temperature-sensitive *prsA* mutants are suppressed by wild-type *ssrA* but not by *ssrA* mutants (24), suggesting a genetic interaction of some type. We offer two highly speculative roles for PrsA in the SsrA·SmpB complex. First, because PrsA can bind a ribose sugar and activate the C-1 carbon for addition of a purine base, it might play some role in recruiting the SsrA·SmpB complex to rare apurinic sites in mRNAs on stalled ribosomes and perhaps even play a role in repairing these damaged sites. Second, under conditions in which the precursors for nucleotides or amino acid synthesis are scarce, PrsA might increase the activity of the SsrA·SmpB system to allow it to deal with enhanced levels of incomplete transcription and/or translation.

The final protein that we identified as being associated with SsrA·SmpB was encoded by the *yfbG* gene. Neither this gene nor its protein product, which we call SAF, have been characterized, although sequence homology suggests the presence of one domain similar to methionine tRNA formyl-transferase and another domain similar to sugar dehydratases or epimerases. One intriguing possibility is that the formyl-transferase domain of SAF formylates the α-amino group of alanyl–SsrA to produce formyl–alanyl–SsrA. Because a free amino group is required for alanyl–SsrA to add the first alanine of the SsrA peptide tag, formylation would seem to play a role in negatively regulating SsrA·SmpB activity. Obvious orthologs of the formyl-transferase domain of SAF are present only in a few bacteria, which is more consistent with a regulatory function than a fundamental role in processes central to the tmRNA mechanism.

Inspection of Fig. 2*B* shows that most of the proteins discussed here are present in sub-stoichiometric quantities relative to SmpB at both the Ni^{2+}-NTA and S300 stages of complex purification. Although this result could indicate the presence of multiple copies of SmpB in the complex, it is more likely that it indicates a deficiency of the other proteins. This deficiency is likely to be a consequence of overproduction of SsrA and SmpB in the cells used for purification and/or loss of less tightly bound components during purification. Nevertheless, it will be necessary to develop methods for isolation of more homogeneous complexes before physical characterization becomes worthwhile. There are also several proteins in the complex, the identities of which have yet to be determined (unlabeled bands in Fig. 2*B* gel, lane 1). Finally, determining how S1, RNase R, PrsA, and SAF support SsrA·SmpB activity, or indeed whether they do, remains a significant challenge.

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