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SsrA-mediated peptide tagging caused by rare codons and tRNA scarcity

Eric D.Roche and Robert T.Sauer[1]

Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

[1]Corresponding author
e-mail: bobsauer@mit.edu

SsrA RNA mediates the addition of a C-terminal peptide tag (AANDENYALAA) to bacterial proteins translated from mRNAs without in-frame stop codons. This process involves both tRNA- and mRNA-like functions of SsrA and targets the tagged proteins for degradation. By designing an SsrA variant that adds a peptide tag (AANDENYALDD) that does not result in rapid degradation, we show that tagging of a model protein synthesized from an mRNA without stop codons can be detected both *in vivo* and *in vitro*. We also use this assay to demonstrate that ribosome stalling at clusters of rare arginine codons in mRNA is sufficient to recruit and activate the SsrA peptide tagging system. An essential requirement for tagging at rare AGA codons is a scarcity of the cognate tRNA; supplemental $tRNA^{AGA}$ suppresses tagging, and depleting the available pool of $tRNA^{AGA}$ enhances tagging and reveals tagging caused by single rare AGA codons. Protein tagging at sites corresponding to rare codons appears to involve SsrA action at an internal mRNA site rather than at the 3′ end of a cleaved mRNA.

Keywords: 10Sa RNA/mRNA/protein degradation and modification/ribosome stalling/tmRNA

Introduction

Cells utilize a variety of quality control systems to deal with the errors that arise during the biosynthesis of macromolecules. In *Escherichia coli*, the SsrA system facilitates the destruction of incomplete proteins expressed from cleaved or broken messages lacking in-frame stop codons (Keiler *et al.*, 1996). This is accomplished by attaching a C-terminal peptide sequence, AANDENYALAA, that targets the tagged protein for degradation (Tu *et al.*, 1995; Keiler *et al.*, 1996; Gottesman *et al.*, 1998; Herman *et al.*, 1998). SsrA is an RNA molecule also known as 10Sa RNA or tmRNA and has properties of both an alanine-tRNA and mRNA (Komine *et al*., 1994, 1996; Ushida *et al.*, 1994; Tu *et al.*, 1995; Tadaki *et al.*, 1996; Williams and Bartel, 1996; Felden *et al.*, 1997, 1998). To explain how SsrA mediates tagging of proteins expressed from mRNAs lacking a stop codon, Keiler *et al.* (1996) proposed the model shown in Figure 1. A ribosome translates to the 3′ end of a message and stalls because it is unable to bind the release factors necessary for termination. Alanine-charged SsrA RNA binds to this stalled ribosome, mimicking a tRNA, and the nascent chain is transferred to alanyl-SsrA by transpeptidation. An unusual mRNA switch then occurs to the AANDENYALAA reading frame within SsrA. This short tag-encoding segment is translated until a stop codon is reached, and the tagged protein is released and subsequently degraded. The tagging function of SsrA also requires SmpB, a protein that binds SsrA RNA and facilitates its association with ribosomes (Karzai *et al.*, 1999).

An important biological role for SsrA is indicated by its presence in almost all prokaryotic chromosomes, including genomes such as *Mycoplasma genitalium* which contain <500 genes (Williams, 1999). This role presumably includes mediating the degradation of partial translation products that might otherwise be detrimental to the cell because of unregulated activities, dominant-negative effects or improper folding (Keiler *et al.*, 1996). In addition, SsrA may release ribosomes from mRNA when translation can not be completed (Keiler *et al.*, 1996; Withey and Friedman, 1999). However, SsrA-tagging has only been documented for one condition, when ribosomes stall at the 3′ end of mRNAs lacking in-frame termination codons. Knowing whether the SsrA system is also employed to deal with other types of translational failures is fundamental to any real understanding of its biological function. Moreover, if SsrA can assist with a wider range of translational defects, then it becomes important to understand what features of such events lead to its recruitment.

Here we describe a sensitive assay for SsrA-mediated tagging and demonstrate that tagging can occur at protein positions encoded by the rare arginine codons AGA and CGA. In *E.coli*, these codons are among the least frequently used (0.26 and 0.36%, respectively; Nakamura *et al*., 1999). Moreover, $tRNA^{AGA}$ is present at low levels in the cell and the tRNA that recognizes CGA translates this triplet inefficiently (Ikemura, 1981; Saxena and Walker, 1992; Emilsson *et al.*, 1993; Curran, 1995). Rare codons have previously been found to slow translation and to cause translational errors, particularly when present as clusters (Robinson *et al.*, 1984; Misra and Reeves, 1985; Bonekamp and Jensen, 1988; Kane, 1995). We find that SsrA-mediated tagging at rare AGA codons is caused by a scarcity of the cognate tRNA, suggesting that ribosome stalling leads to activation of the SsrA system.

Results

An assay for SsrA tagging

C-terminal addition of the SsrA tag, AANDENYALAA, is difficult to detect because the tagged polypeptide is degraded. It is known, however, that proteins ending with the gene-encoded sequence AANDENYALDD are relatively stable (Keiler *et al.*, 1996; Gottesman *et al.*, 1998; Herman *et al.*, 1998). We therefore constructed a

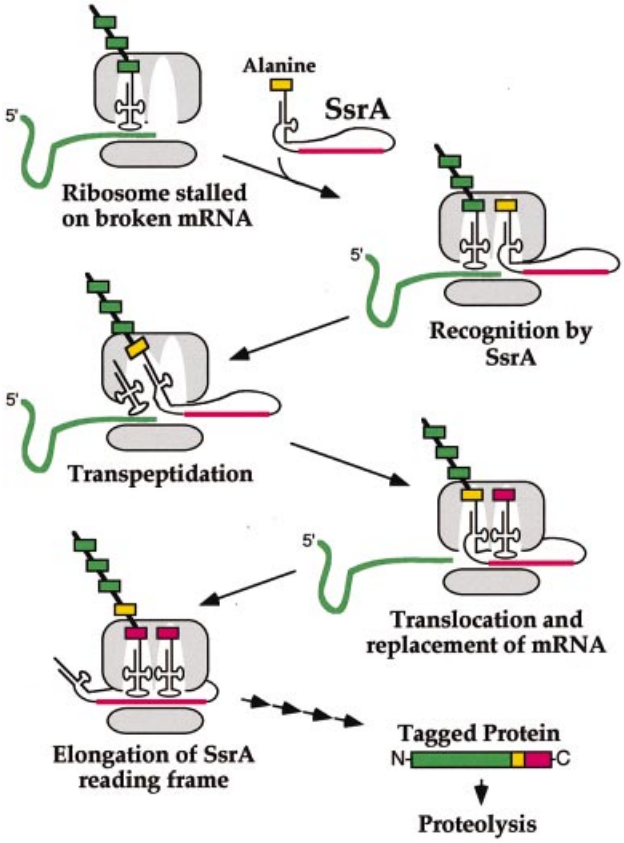

Fig. 1. Model for SsrA-mediated tagging of proteins synthesized from messages lacking stop codons (Keiler *et al.*, 1996). See text for details.

mutant, SsrA-DD, in which the final two codons of the peptide reading frame were changed to encode aspartic acids (Figure 2A). In principle, the SsrA-DD tagging reaction should add the peptide AANDENYALDD to appropriate targets and the resulting tagged proteins should be relatively resistant to degradation. To detect DD-tagged proteins, polyclonal antibodies against a synthetic AANDENYALDD peptide were raised and affinity purified. These purified anti-DD antibodies were highly specific for proteins with the SsrA-DD tag and showed no cross reactivity with proteins bearing the wild-type AA tag or with untagged bacterial proteins (data not shown).

To confirm that SsrA-DD RNA is active, we examined tagging by this RNA in cells lacking wild-type SsrA and containing λN-trpAt mRNA, a message that has no in-frame stop codon and that encodes the N-terminal domain of λ repressor (Figure 2B; Keiler *et al.*, 1996). Western blots of lysates were developed using anti-DD antibodies and showed a band corresponding to DD-tagged λN-trpAt protein that increased in level with time after induction of the λN-trpAt gene (Figure 2C, lanes c–e). This tagged product was absent if either the SsrA-DD gene or the λN-trpAt gene was not present (Figure 2C, lanes a and b). Thus, SsrA-DD RNA is active in tagging *in vivo* and this RNA can be used in combination with antibodies to the DD tag to assay tagging. However, as judged by levels of untagged λN-trpAt protein, tagging mediated by the multicopy, plasmid-borne *ssrA-DD* gene in an *ssrA*-deletion strain was only 50–70% as efficient as tagging mediated by the single chromosomal *ssrA* gene in a wild-type strain (data not shown). As a result, assays performed using SsrA-DD RNA probably underestimate the extent of wild-type tagging.

DD-tagged cellular proteins were also observed in these assays (Figure 2C, lanes b and c). These proteins cross reacted with the anti-DD antibodies, had higher molecular weights than the DD-tagged λN-trpAt protein, were absent in cells lacking SsrA-DD (Figure 2C, lane a), and were present prior to induction of the λN-trpAt gene (Figure 2C, lane c) and in cells lacking the λN-trpAt gene (Figure 2C, lane b). The SsrA-DD assay therefore allows detection and studies of endogenous tagging of proteins in *E.coli*. Intriguingly, the steady-state levels of these DD-tagged cellular proteins decreased rapidly after induction of the λN-trpAt gene (Figure 2C, lanes d and e), suggesting that λN-trpAt mRNA competes efficiently with cellular messages for SsrA-DD tagging.

SsrA-DD RNA also provides a direct means of assaying tagging *in vitro*. SsrA-DD RNA was synthesized by transcription *in vitro* with T7 RNA polymerase and added to an S30 transcription/translation reaction programmed with λN-trpAt DNA. A tagged product of the expected size was detected on Western blots probed with anti-DD antibodies (Figure 2D, lane c) but was absent if either λN-trpAt DNA or SsrA-DD RNA was omitted (Figure 2D, lanes a and b). As a result, SsrA-DD RNA transcribed *in vitro* is active in tagging, indicating that any necessary RNA folding, base modifications, or binding of cofactors such as SmpB can also occur *in vitro*. Indirect evidence for SsrA-directed tagging *in vitro* has been reported (Hanes and Pluckthun, 1997; Himeno *et al.*, 1997).

Tagging induced by a rare-codon cluster

Can ribosome stalling within an mRNA result in SsrA-tagging? To address this question, we constructed a gene (λN-4AGA) encoding a His_6 tag, the N-terminal domain of λ repressor, a segment containing four consecutive arginines (encoded by four rare AGA codons), an M2 Flag epitope and a translation termination codon (Figure 3A). Four AGA codons were used because clusters of rare codons are known to slow translation significantly (Robinson *et al.*, 1984; Misra and Reeves, 1985; Bonekamp and Jensen, 1988).

Anti-DD-probed Western blots of lysates from *ssrA*-deficient cells containing SsrA-DD and the λN-4AGA gene showed a band of the size expected for a protein truncated and tagged at or near the rare-codon region (Figure 3C, lanes b–d). Expression of this product depended on the presence of the λN-4AGA gene and SsrA-DD (Figure 3C, lanes e and f) and upon induction of the λN-4AGA gene (Figure 3C, lane a). The principal tagged species was purified by Ni^{2+}-NTA chromatography, anion-exchange chromatography and reverse-phase chromatography. The purified protein was analyzed by MALDI/TOF mass spectrometry and had the mass expected if the tag was added instead of the first arginine encoded by the four AGA cluster (Figure 3B and D). Moreover, this protein contained the His_6 and DD-tag epitopes but not the M2 Flag epitope (data not shown), as expected if normal translation stops and tagging occurs at the run of rare codons. These experiments show that SsrA-mediated tagging can be induced by a string of rare codons. Tagging of cellular proteins was also observed in this experiment

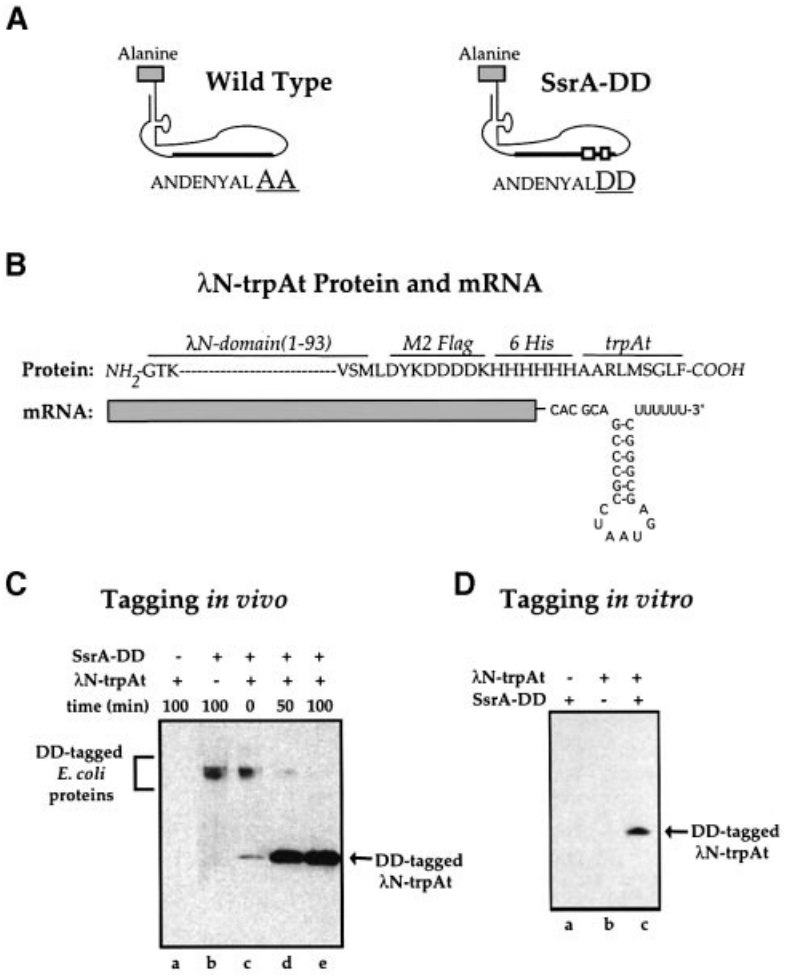

Fig. 2. Development and testing of an assay for SsrA-tagging. (**A**) SsrA-DD RNA contains base mutations that change the final two amino acids of the peptide tag from alanines to aspartic acids. (**B**) λN-trpAt protein (containing residues 1–93 of the N-terminal domain of λ repressor, an M2 Flag epitope, a His_6 tag, and residues encoded by the trpAt transcriptional terminator) is encoded by an mRNA with no in-frame termination codons. (**C**) SsrA-DD tagging of λN-trpAt protein *in vivo*. Strain X90 *ssrA::cat* transformed with pKW22 and pPW500 (lanes c–e), pKW22 and pAD100 (lane b), or pKW1 and pPW500 (lane a) was grown to mid-log phase at 37°C and induced with IPTG at time zero. Samples were removed at the times indicated and analyzed by Western blotting using antibodies to the AANDENYALDD peptide tag (anti-DD). (**D**) SsrA-DD tagging of λN-trpAt protein *in vitro*. Unmodified, mature SsrA-DD RNA and DNA encoding λN-trpAt were added together or individually to *E.coli* S30 transcription/translation reactions. After incubation for 30 min at 37°C, reactions were analyzed by Western blotting using anti-DD antibodies.

(Figure 3C, lanes a and e), and λN-4AGA mRNA competed with cellular messages for tagging (Figure 3C, lanes b–d) as seen previously with λN-trpAt mRNA, which lacks stop codons. In addition, SsrA-DD-mediated rare-codon tagging, like tagging of mRNAs without stop codons, was absent in strains lacking SmpB protein (data not shown; Karzai *et al.*, 1999), suggesting that both reactions share common mechanistic features. Finally, the SsrA-DD-tagged λN-4AGA protein was present in cell lysates at roughly one-quarter the level of full-length λN-4AGA protein, indicating that SsrA-DD-mediated tagging at the 4AGA rare-codon cluster represents a significant fraction of the protein produced under the conditions of this experiment.

Requirements for rare-codon tagging

To determine the number of rare codons needed to induce tagging, we designed genes analogous to λN-4AGA but with one or two AGA codons (Figure 4A). Each of these constructs contains four consecutive arginine codons, with the common CGC codon used as the alternative to the rare AGA codon. When the different λN-AGA proteins were expressed in the presence of SsrA-DD RNA, tagging was observed at the clusters of two or four AGA codons (Figure 4B, upper panel, lanes e and f) but not at the single AGA codon (Figure 4B, upper panel, lane d). Thus, the minimum requirement for detectable tagging under these conditions is two contiguous AGA codons. The λN-2AGA and λN-4AGA proteins from these experiments were purified by Ni^{2+}-NTA chromatography and were shown to include a species corresponding to DD-tagging at the first AGA codon as detected by mass spectrometry (data not shown). Therefore, tagging at rare AGA codons occurs preferentially at the first AGA but requires the presence of subsequent AGA codons.

Western blots probed with antibodies to the His_6 tag revealed substantially lower expression of the full-length λN-4AGA protein than the λN-1AGA and λN-2AGA proteins, and showed that the major tagged λN-4AGA product was present at ~20% of the level of the full-length protein (Figure 4B, lower panel, lanes d–f). Other minor tagged and untagged species with lower molecular

weights than the full-length protein were also present for both λN-2AGA and λN-4AGA (Figure 4B, lanes e and f). The reduced expression and the production of untagged, lower molecular weight species due to frameshifting or premature termination is consistent with results from other systems examining the effects of rare codons (Spanjaard and Duin, 1988; Gurskii *et al.*, 1992; Rosenberg *et al.*, 1993). However, among the protein products detected in our experiment, SsrA-mediated rare-codon tagging was the primary alternative to normal translation.

If SsrA-DD tagging at rare AGA codons results from ribosome stalling, then this tagging should be suppressed by overproduction of $tRNA^{AGA}$. To enable studies of this type, a plasmid that expresses both SsrA-DD RNA and $tRNA^{AGA}$ was constructed. Overexpression of $tRNA^{AGA}$ prevented tagging of both the λN-2AGA and λN-4AGA gene products (Figure 4B, upper panel, lanes b and c), indicating that ribosome stalling caused by a scarcity of free $tRNA^{AGA}$ is a prerequisite for SsrA-mediated tagging of these substrates.

SsrA-tagging induced by depletion of a tRNA pool

To determine whether depletion of free $tRNA^{AGA}$ results in enhanced SsrA-DD tagging at rare AGA codons, we examined the effect of inducing an mRNA encoding a short open reading frame (ORF) with eight consecutive AGA codons. Plasmids were constructed expressing the eight AGA ORF under arabinose control and a second message under isopropylthio-β-D-galactopyranoside (IPTG) control encoding a λN-0AGA, λN-1AGA or λN-2AGA protein (Figure 5A). These constructs and control plasmids without the eight AGA ORF were assayed for SsrA-DD tagging. Western blot analysis showed that induction of the eight AGA ORF resulted in tagging of the λN-1AGA protein (Figure 5B, upper panel, lane e). The major tagged species was expressed at ~10% of the level of the highly expressed, full-length protein (Figure 5B, lane e). Constructs containing no AGA codons or containing one AGA codon but lacking the eight AGA ORF exhibited no SsrA-DD tagging (Figure 5B, upper panel, lanes a, b and d). The λN-2AGA protein was tagged

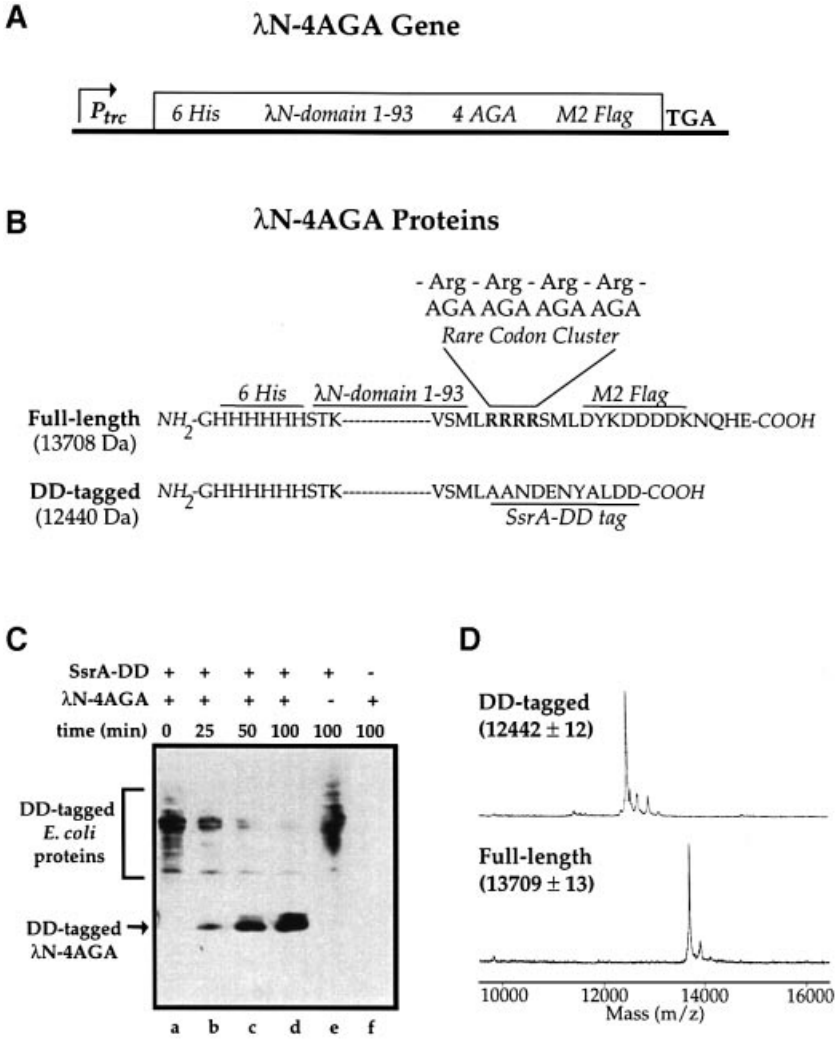

Fig. 3. Tagging detected at rare AGA codons. (**A**) Design of the λN-4AGA gene containing four consecutive AGA arginine codons. This gene contains an in-frame stop codon prior to a transcriptional terminator. (**B**) Sequence and expected masses of the full-length and the principal DD-tagged λN-4AGA proteins. (**C**) Tagging of λN-4AGA protein. Strain X90 *ssrA::cat* transformed with plasmid pairs pKW22 and pER118-1 (lanes a–d), pKW22 and pAD100 (lane e), or pKW1 and pER118-1 (lane f) was grown at 37°C, induced at an OD_{600} of 0.5–0.6, and analyzed after various times by Western blotting using anti-DD antibodies. (**D**) MALDI/TOF mass spectra of the full-length and the principal DD-tagged λN-4AGA proteins.

in the presence and absence of the eight AGA ORF. However, when the eight AGA ORF was expressed the extent of tagging was greater (Figure 5B, upper panel, lanes c and f), there was reduced expression of full-length protein (lower panel, lanes c and f) and substantial quantities of smaller, untagged species accumulated (lower panel, lane f). The occurrence of SsrA-DD tagging at a single AGA codon suggests that the only significant requirement for rare-codon tagging is a scarcity of the corresponding tRNA and that expression of mRNAs containing repeated AGA codons causes tagging largely by reducing free tRNA levels.

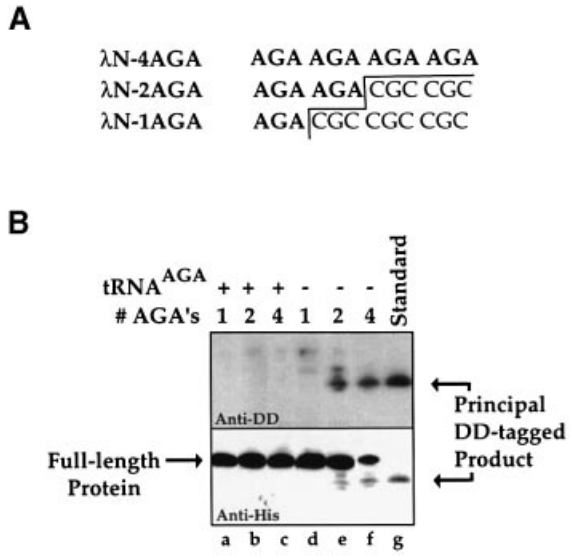

Fig. 4. SsrA-tagging at AGA clusters of varying size is suppressed by expression of $tRNA^{AGA}$. (**A**) Constructs similar to λN-4AGA (Figure 3A) but expressing λN-AGA genes with one, two or four rare AGA arginine codons and three, two or zero common CGC arginine codons. (**B**) Western blot analysis of proteins with one (pER156), two (pER157) or four (pER158) AGA codons in the presence (pER203) or absence (pKW23) of supplemental $tRNA^{AGA}$. Strain X90 *ssrA::cat* containing different plasmid pairs was grown to mid-log phase at 37°C, induced with IPTG, and grown for an additional 2 h. The standard is purified SsrA-DD-tagged λN-4AGA protein.

Tagging caused by CGA codons

Is SsrA-mediated rare-codon tagging observed at codons other than AGA? To test this, tagging of a λN-AGA-3CGA construct, which contained rare CGA codons rather than common CGC codons at positions 2–4 (Figure 6A), was compared with tagging of λN-4AGA in cells over-expressing $tRNA^{AGA}$. Under these conditions, AGA becomes a 'common' codon. Multiple DD-tagged proteins were observed for λN-AGA-3CGA (Figure 6B, lanes b and d), but no tagged proteins were observed for λN-4AGA (Figure 6B, lanes a and c). MALDI/TOF mass spectrometry of the His-tagged λN proteins from this experiment showed peaks for proteins tagged at each CGA position, but in descending intensity from the third to the first CGA codon (Figure 6C).

Characterization of rare-codon mRNA

SsrA-mediated tagging at rare codons appears to involve SsrA recruitment to a ribosome stalled within an mRNA, a mode of tagging that has not been described previously. Alternatively, for tagging at rare codons to be explained by the SsrA-tagging model shown in Figure 1, a truncated mRNA species ending at or within the first rare codon would have to be generated by mRNA cleavage or premature termination of transcription. To assay for an mRNA species of this type, we purified total RNA from cells containing SsrA-DD RNA and a λN-AGA gene (one, two or four AGA codons), with or without additional $tRNA^{AGA}$. Tagging under these conditions occurs only in the presence of two or four AGA codons and in the absence of additional $tRNA^{AGA}$. If mRNA cleavage is indeed the cause of tagging, then messages ending at the first AGA codon should also appear only in these samples. Northern blots probed with a DNA oligonucleotide complementary to a 5′ portion of the mRNA showed no truncated mRNA species of the expected length under any of the conditions examined (Figure 7). However, higher steady-state levels of the full-length message were observed for both conditions that result in SsrA-DD tagging (Figure 7, lanes d and e). This increase in message level may result from ribosome stalling at the rare codons and a subsequent back-up of other ribosomes that protects

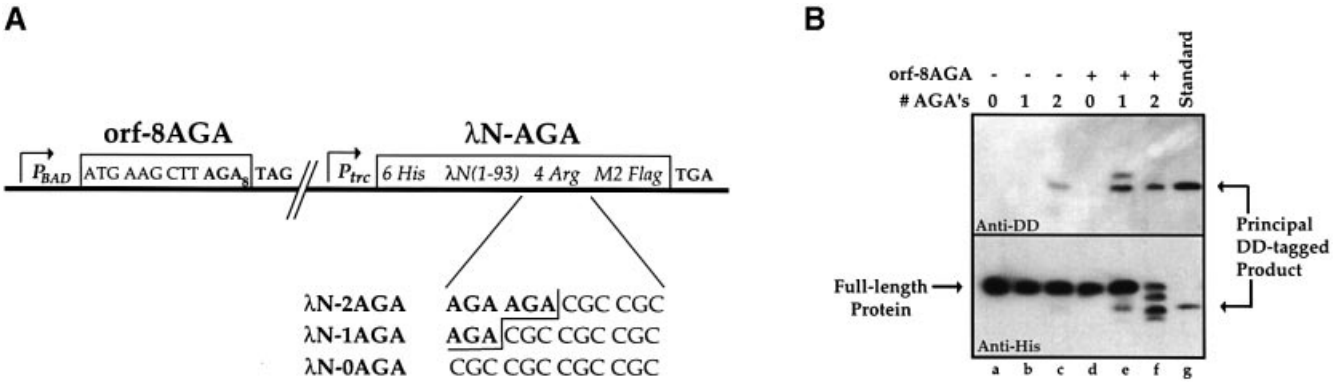

Fig. 5. Depletion of $tRNA^{AGA}$ enhances SsrA-tagging at single and tandem AGA codons. (**A**) A short ORF containing eight AGA codons under arabinose promoter control was used to deplete the free pool of $tRNA^{AGA}$. λN-AGA proteins were synthesized from the λN-0AGA, λN-1AGA or λN-2AGA genes with the indicated number of AGA codons. (**B**) Western analysis of the effects of the eight AGA ORF. Strain X90 *ssrA::cat* cells contained plasmid pKW23 and either pER154 (lane a), pER156 (lane b), pER157 (lane c), pER174 (lane d), pER176 (lane e) or pER177 (lane f). Experiments were performed as described in the Figure 4 legend except the eight AGA ORF was induced by addition of 2% arabinose 15 min before induction of the λN-AGA genes with IPTG.

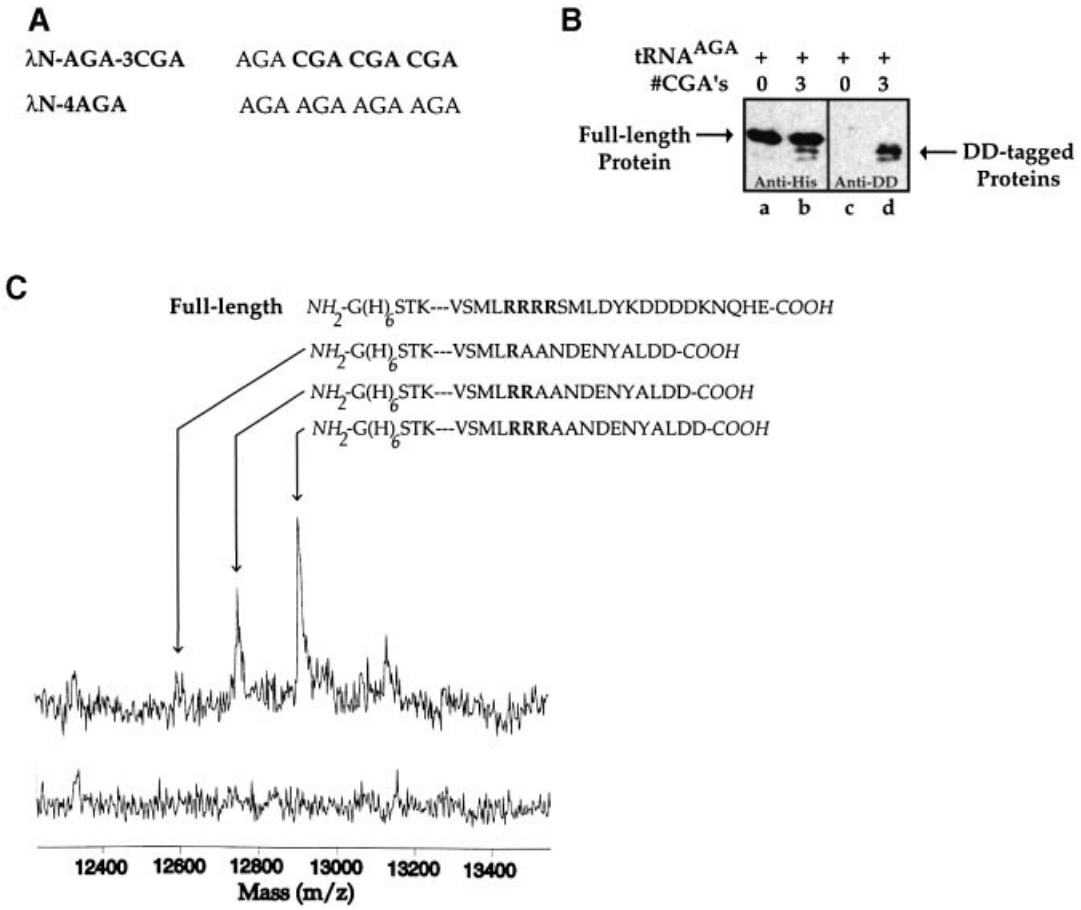

Fig. 6. Tagging at CGA codons. (**A**) Rare codon cluster in the λN-AGA-3CGA gene, which is otherwise identical to λN-4AGA (see Figure 3A). (**B**) Tagging of the λN-AGA-3CGA and λN-4AGA genes in cells overexpressing $tRNA^{AGA}$. Cell lysates of strain X90 *ssrA::cat* transformed with plasmid pER203 and either pER118-1 (lanes a and c) or pER106 (lanes b and d) were analyzed by Western blotting. Cells were grown and induced as described in Figure 4. (**C**) MALDI/TOF mass spectra of proteins translated from messages containing three CGA codons (upper trace) or no CGA codons (lower trace). The arrows mark the expected masses of proteins tagged at the first, second and third CGA codons.

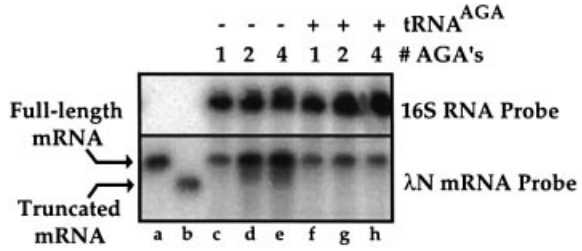

Fig. 7. Northern blot analysis of λN-AGA mRNA. Lanes a and b contain control mRNAs transcribed *in vitro* and corresponding to full-length message (3 ng) and a message truncated at the first AGA (2.4 ng), respectively. Lanes c–h each contain 1.5 μg of total cellular RNA from the experiments analyzed by Western blotting in Figure 4. Samples were electrophoresed on a 2.8% agarose formaldehyde gel, transferred to a membrane and probed with ^{32}P-labeled oligonucleotides complementary either to a 5′ segment of the λN-AGA mRNA or a portion of 16S ribosomal RNA.

the message from degradation. Previous studies have also reported that the presence of rare codons can increase the steady-state level of a message (Ivanov *et al.*, 1997).

RNase protection assays were also performed to search more sensitively for messages ending at the first AGA codon under conditions resulting in tagging. RNA probes complementary to the 3′ portion of each λN-AGA message were synthesized for these experiments (Figure 8A). Control messages synthesized *in vitro* corresponding either to the full-length mRNA or a truncated message ending at the first AGA codon (Figure 8A) were detected effectively by this assay (Figure 8B, lanes a and b). Mixing experiments also showed that a truncated control message corresponding to ~2% of the full-length message in cellular samples could be detected (Figure 8B, lanes c, e and h). Samples of total cellular RNA from cells in which DD-tagging occurs did contain truncated RNA (Figure 8B, lanes f and i) but only at levels slightly higher than samples from cells in which DD-tagging does not occur (Figure 8B, lanes g and j). The quantity of truncated message in cells displaying tagging was <2% of the full-length message. The product expected from mRNA cleavage at the first AGA codon also represented only a small fraction of the total set of incomplete mRNA fragments (Figure 8B, lanes f and i). These results suggest that tagging at rare codons proceeds by SsrA action at an internal mRNA site rather than at the 3′ end of a cleaved message.

Discussion

We have developed a simple and sensitive assay for SsrA-mediated tagging that detects tagging of a model protein translated from an mRNA without in-frame stop codons both *in vivo* and *in vitro*. Using this assay, we have also

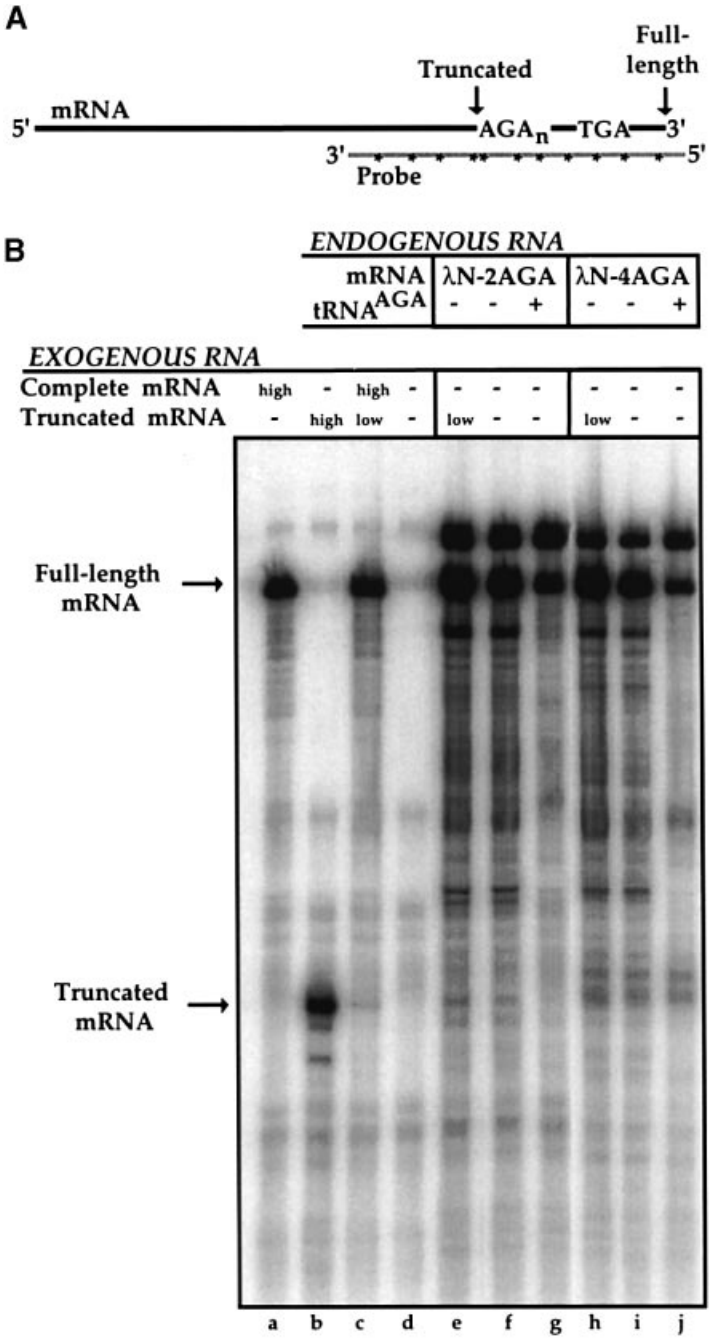

Fig. 8. RNase protection of λN-AGA messages. (**A**) A ribonucleotide probe complementary to the 3′ portion of each λN-AGA message and extending ~30 bases beyond the 3′ end of each message was synthesized using [α-^{32}P]UTP. Control messages were also synthesized corresponding either to mRNA truncated at the first rare codon (a fragment that is identical for all λN-AGA mRNAs) or to the full-length λN-2AGA message. (**B**) Results of the protection assay. The probes were hybridized to control and/or cellular RNAs, digested with RNases A and T1 to remove single-stranded regions and electrophoresed on a 6% sequencing gel (National Diagnostics). Control lanes (a–d) used the λN-2AGA probe. The lanes marked λN-2AGA or λN-4AGA contained 0.6 μg of total RNA from the experiments in Figure 4 with cells expressing the products of these genes in the presence or absence of supplemental tRNA^{AGA}. Note that DD-tagging of the λN-2AGA and λN-4AGA proteins is observed only in the absence of supplemental tRNA. Full-length and truncated control mRNAs were added at high levels (1.6 μg) or low levels (60 pg) to the samples indicated. All samples included 9 μg of yeast tRNA. Lane d shows digestion of the probe in the absence of control and cellular mRNAs. In the presence of cellular RNA, there is some protection of the full-length probe resulting in a band above that corresponding to the full-length message.

demonstrated that proteins synthesized from messages containing rare codons can be tagged by the SsrA system. SsrA-mediated tagging was detected at both rare AGA and CGA arginine codons. A significant preference was observed for SsrA-mediated tagging at sites corresponding to the first rare codon in an AGA cluster, whereas tagging occurred preferentially at later positions in a CGA cluster. The reason for this difference is unknown but may involve differences in the cellular level of the cognate tRNAs, in their decoding efficiencies and/or in the effects of P-site tRNA on translation in the A site (Ikemura, 1981; Saxena and Walker, 1992; Emilsson *et al.*, 1993; Curran, 1995; Mejlhede *et al*., 1999). Scarcity of the cognate tRNA is a clear requirement for tagging induced by rare AGA codons.

Increasing the concentration of $tRNA^{AGA}$ eliminates tagging at clusters of rare AGA codons, whereas decreasing the free tRNA concentration results in a greater extent of tagging and causes tagging at single rare AGA codons. Taken together, these results indicate that repeated rare AGA codons cause tagging by depleting the available pool of tRNA.

How does SsrA-mediated tagging occur when ribosomes stall at rare codons? For tagging to proceed by the model shown in Figure 1, ribosome stalling would need to result in cleavage of the mRNA at the rare codons. Following dissociation of the untranslated RNA fragment, this would result in a ribosome at the 3′ end of an mRNA fragment. This mechanism can not be eliminated but seems unlikely for several reasons. For example, under conditions where the DD-tagged protein is the major modified product and is present at ~20% of the level of the full-length protein, mRNA of the size expected for cleavage at the AGA cluster comprises no more than 2% of the level of the complete message and represents only a small fraction of the incomplete or cleaved fragments of this mRNA. Furthermore, ribosomes have been shown to protect ~30 bases of mRNA, centered on and including the P and A sites, from ribonuclease and chemical cleavage (Huttenhofer and Noller, 1994, and references therein). Thus, if tagging did occur by an mRNA cleavage mechanism, then cleavage of a stalled mRNA within the A site would require a novel endonucleolytic activity, presumably catalyzed by the ribosome itself. An alternative model is that ribosome stalling at rare codons directly results in SsrA recruitment and peptide tagging at internal sites of a complete message. By this model, which is more consistent with our results, SsrA binding and message switching would occur despite the presence of a substantial region of untranslated 3′ mRNA.

Rare codons, especially in clusters and when the cognate tRNA is limiting, have previously been shown to cause a variety of translational defects including premature termination, frameshifting, ribosomal hopping and misincorporation (Spanjaard and van Duin, 1988; Spanjaard *et al.*, 1990; Gurskii *et al.*, 1992; Kane *et al.*, 1992; Rosenberg *et al.*, 1993; Calderone *et al.*, 1996). It is not surprising that SsrA-tagging at rare codons has not been observed previously, as proteins tagged by wild-type SsrA are rapidly degraded and would not normally be detected. In our experiments with clusters of AGA codons, the SsrA-DD-tagged protein was the principal alternative product to full-length protein. Moreover, when tagging was observed at single rare codons because of depletion of the cognate tRNA, significant levels of other incomplete translation products were not detected. In fact, substantial levels of untagged, alternative products were only observed for clusters of AGA codons when $tRNA^{AGA}$ was depleted, possibly because frameshifting requires repeated rare codons and is more likely to occur under these circumstances. When expressing protein from an mRNA containing an AGA codon cluster, we also observed the accumulation of a truncated protein product in cells lacking SsrA that did not appear when either SsrA-DD or wild-type SsrA was present (data not shown). However, similar levels of full-length protein were produced in $SsrA^+$ and $SsrA^-$ cells under these conditions. These observations suggest that SsrA tagging acts to prevent accumulation of truncated products when ribosomes stall rather than to increase overall synthesis of full-length protein.

SsrA-DD tagging occurs at sites of rare AGA codon clusters under normal growth conditions and at single rare AGA codons when the cognate tRNA is partially depleted. The efficiency of this tagging is ~5–25% relative to translational read-through. We note, however, that SsrA-DD is less active than wild-type SsrA in tagging proteins expressed from mRNAs without stop codons, and it is possible that this may also be true for rare-codon tagging. The major function of SsrA in relation to rare codons is probably to rid the cell of potentially noxious protein fragments and/or to free ribosomes stalled at these sites. An analysis of the *E.coli* genome reveals that only 72 genes contain AGG-AGG, AGA-AGA or CGA-CGA rare-codon clusters and two genes contain AGA-AGA-AGA or CGA-CGA-CGA sequences. Thus, under normal growth conditions, we anticipate that a relatively small number of mRNAs would experience ribosome stalling at rare codons with subsequent SsrA-mediated tagging. In contrast, 49% of *E.coli* genes contain at least one rare AGA or AGG codon, so that even a low level of stalling-induced tagging at these sites could result in a substantial quantity of tagged proteins. Stalling at single rare codons might also become important during stationary phase or under other nutrient-limited conditions in which scarce tRNAs become limiting for translation. Increased expression of $tRNA^{AGA}$ enhances production of a variety of cellular proteins only in stationary phase, suggesting that this rare tRNA does become limiting under these conditions (Chen and Inouye, 1994). The association of SsrA RNA with 70S ribosomes also increases in stationary phase, as do SsrA levels in some bacteria (Komine *et al.*, 1996; Watanabe *et al.*, 1998). Intriguingly, SsrA-defective cells are more deficient in growth during the approach to stationary phase than in log phase, and the pattern of tagged cellular proteins changes as bacteria approach stationary phase (K.Williams and E.D.Roche, unpublished).

SsrA-mediated peptide tagging has now been detected in two distinct situations, when ribosomes stall at rare codons and when ribosomes reach the 3′ end of an mRNA. The results presented here indicate that tagging is induced when ribosomes stall. Thus, SsrA may also function to avoid the problems of truncated protein fragments and stalled ribosomes for a more extensive set of translational problems. For example, tagging might occur at untranslatable sites of RNA damage, during the stringent response when a shortage of amino acids leads to a deficiency of charged tRNAs, if ribosomes stall at stop codons when release factors are scarce, or when secondary structures in an mRNA lead to a slowing or pausing of translation. Some mRNAs may even have evolved sequences to induce tagging under specific conditions, allowing expression of these genes to be regulated via tagging and degradation. Finally, *Mycoplasma capricolum* contains an unassigned codon that does not occur in wild-type reading frames, has no cognate tRNA and is not a stop codon (Oba *et al.*, 1991). SsrA function in this bacterial species could bypass the deleterious effects of single mutations or frameshifts that stall ribosomes at this untranslatable codon. The *ssrA* gene is not essential in *E.coli* (Oh and Apirion, 1991). Nevertheless, the almost universal presence of SsrA RNA

in Gram-negative and Gram-positive prokaryotes, including species with remarkably small genomes, suggests an important role in translational quality control and ribosome management that confers a significant evolutionary advantage to these bacteria (Williams, 1999).

Materials and methods

Strains and plasmids

Escherichia coli strain X90 *ssrA::cat* and plasmid pPW500, which expresses the λN-trpAt protein, have been described previously (Keiler *et al*., 1996), as have strains W3110 *ssrA::kan* and W3110 Δ*smpB* (Komine *et al*., 1994; Karzai *et al*., 1999). Plasmid pKW22 has a p15A origin of replication, encodes tetracycline resistance and expresses SsrA-DD RNA (in which the last two codons of the peptide-tag sequence are changed from GCA GCT to GAT GAT) from the wild-type *ssrA* promoter; pKW23 contains additional compensating mutations that maintain base-pairing in the P5 stem (Williams and Bartel, 1996) but is otherwise identical to pKW22. Plasmids pKW22 and pKW23 were constructed in collaboration with Kelly Williams and were roughly equivalent in the extent of DD-tagging of the λN-trpAt protein (data not shown). Plasmid pKW1 has the same backbone as pKW22 but lacks the *ssrA* gene. Except as indicated, bacterial cultures and colonies were grown at 37°C in Luria–Bertani (LB) broth or on LB agar.

Plasmid pER203 expresses SsrA-DD RNA and $tRNA^{AGA}$, with the latter gene under control of the Pro_1 tRNA promoter and terminator (Komine *et al*., 1990). This plasmid was constructed by PCR amplification of the $tRNA^{AGA}$ sequence using primers 5′-CCC GGG GCT AGC AAG GGA TTG ACG AGG GCG TAT CTG CGC AGT AAG ATG CGC CCC GCA TTG CGC CCT TAG CTC AGT TGG AT-3′ and 5′-CCC GGG GCT AGC AAA AAA GCC TGC TCG TTG AGC AGG CTT TTC GAA TTT GGC GCG CCC TGC AGG ATT C-3′. The resulting product was digested with *Nhe*I and ligated to the backbone of plasmid pKW23 which had been partially digested with *Nhe*I and treated with alkaline phosphatase. The structure of pER203 was confirmed by PCR screening, restriction mapping and DNA sequencing. Plasmid pER203 allows overexpression of the λN-4AGA protein, which is normally poorly expressed, and mediates DD-tagging of the λN-trpAt protein as efficiently as pKW23.

Plasmid pER118-1 expresses the λN-4AGA protein under control of an IPTG-inducible P_{trc} promoter and has the backbone of pAD100 (Davidson and Sauer, 1994), a pBR322-based plasmid carrying an ampicillin resistance gene (Ap^R), *lacI*^{q} and an F1 phage origin of replication. This construct includes the sequence 5′-GC ATG CTA AGA AGA AGA AGA AGC ATG C-3′ between two *Sph*I sites following the λ repressor N-terminal domain. Plasmids pER154, pER156, pER157 and pER158 contain a region with four consecutive arginine codons with zero, one, two or four AGA codons, respectively, and four, three, two or zero CGC codons (Figure 4A). Plasmid pER158 is identical to pER118-1 but a *Sph*I site distant from the λ repressor N-terminal domain coding region has been removed by blunting with T4 DNA polymerase and religating, making the *Sph*I sites within the coding segment unique, and enabling construction of plasmids pER154, pER156 and pER157 by insertion of appropriate oligonucleotide cassettes into the *Sph*I digested backbone of pER158. Plasmid pER106 has the same backbone but an arginine codon sequence of AGA CGA CGA CGA. All λ repressor N-terminal domain constructs used in these studies were encoded by an engineered gene that contains no AGA codons (Breyer and Sauer, 1989).

Plasmids containing a short open reading frame with eight AGA codons under control of the P_{BAD} promoter were constructed as follows. Plasmid pER133 was constructed by digesting plasmid pMPM-A5 (Mayer, 1995) with *Nco*I and *Xho*I, and ligating the backbone fragment to the kinased, annealed oligonucleotides 5′-C ATG AAG CTT AGA AGA AGA AGA AGA AGA AGA AGA TAG ATA GAT AGG-3′ and 5′-T CGA CCT ATC TAT CTA TCT TCT TCT TCT TCT TCT TCT AAG CTT-3′. Plasmids pER174, pER176 and pER177 combine the *Alw*NI to *Eco*47III fragment of pER133, including Ap^R, the eight AGA ORF, and *araC*, with the *Alw*NI to *Sma*I fragment of plasmids pER154, pER156 and pER157, including *lacI*^{q} and the λ repressor N-terminal domain reading frame. These plasmids express the λ N-terminal domain protein (with zero, one or two AGA codons) under IPTG control and the eight AGA ORF under arabinose control.

Antibodies and Western analysis

Rabbit polyclonal antibodies against the KLH-conjugated peptide *NH_2*-AANDENYALDD-*COOH* were raised by Peptide Innovations, Inc. To purify these antibodies, excess AANDENYALDD peptide was coupled to Aminolink Plus Coupling Gel (Pierce) according to the manufacturer's protocol, antiserum was loaded onto the column at neutral pH, the column was washed, and specific antibodies were eluted with pH 2.3 buffer and then returned to neutral pH. The purified antibodies were highly specific for the AANDENYALDD tag sequence, showing no cross reaction on Western blots with proteins in normal *E.coli* cell lysates or with proteins bearing the wild-type AANDENYALAA tag.

Anti-FLAG M2 antibody was purchased from Eastman Kodak. His_6 tags were detected with either His-probe H15 (Santa Cruz Biotechnology) or Penta-His antibody (Qiagen). Horse radish peroxidase-conjugated antibodies (sheep anti-mouse and donkey anti-rabbit) were purchased from Amersham.

Samples for Western analysis were removed from cell cultures, placed on ice, pelleted in a microcentrifuge at 4°C and the cell pellets were frozen at –80°C after removal of the supernatant. The thawed pellets were resuspended in 100 ml TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) per 0.75 OD_{600}/ml of culture, and mixed with an equal volume of 2× sample buffer (20% glycerol, 4% SDS, 1.42 M β-mercaptoethanol, 125 mM Tris, pH 8.0 and bromophenol blue). Samples were heated for 5 min at >95°C and then vortexed vigorously for 1 min. Equal volumes (corresponding to equal OD_{600}/ml of culture) were electrophoresed on 15% Tris-tricine gels, analyzed by Western blotting using the DD, M2 Flag, or His_6 primary antibodies with appropriate secondary antibodies, and detected using Amersham's ECL Western blotting detection agents and X-ray film. The film was scanned with Deskscan II software and a Hewlett Packard ScanJet 4C and data were quantified with ImageQuant Version 5.0 software.

RNA synthesis in vitro

SsrA-DD RNA and marker mRNAs were synthesized *in vitro* with T7 RNA polymerase using standard methods (Sampson and Uhlenbeck, 1988). The DNA template for SsrA-DD transcription was made by PCR amplifying plasmid pKW22 with primers 5′-TGG TGG AGC TGG CGG GAG TTG-3′ and 5′-ATT CCG AAT TCT AAT ACG ACT CAC TAT AGG GGC TGA TTC TGG ATT CGA CGG GGG GA-3′. This transcription reaction included 5 mM GMP for initiation of SsrA RNA and 4 mM NTPs. The DNA templates for transcription of the marker mRNAs used in Figures 7 and 8 were made by amplifying plasmid pER157 with primer 5′-CCG AAT TCT AAT ACG ACT CAC TAT AGG GTG AGC GGA TAA CAA TTT CAC-3′ and either 5′-AAA AAA CCC CTC AAG CCC GTT TA-3′ (full-length message) or 5′-TAG CAT GCT AAC CGC TTC ATA C-3′ (truncated message).

Tagging in vivo

A common protocol with minor modifications was used for all tagging experiments *in vivo*. Competent cells of *E.coli* strain X90 *ssrA::cat* were transformed with appropriate pairs of plasmids, individual transformants were picked, and overnight cultures were grown at 37°C in LB media containing 50 μg/ml ampicillin and 20 μg/ml tetracycline. Fresh cultures were started the next day, typically with a 1:500 dilution of the overnight culture, grown to an OD_{600} of ~0.3–0.4, and induced by addition of 1 mM IPTG. Samples were removed to ice immediately prior to the addition of IPTG and at the desired times after induction. One sample of each type was used for OD_{600} measurements and others were analyzed by Western blotting experiments.

Tagging in vitro

The *E.coli* S30 extract system for linear templates (Promega) was used for studies of SsrA-DD tagging *in vitro*. The transcription/translation reactions included 66 ng/μl of plasmid pPW500 linearized with *Nde*I or 0.5 mM of SsrA-DD RNA synthesized *in vitro*, or a combination of both pPW500 and SsrA-DD RNA. Reactions were made according to the manufacturer's protocol, incubated at 37°C for 30 min, and acetone precipitated. Samples were resuspended in 1× SDS sample buffer and analyzed by Western blotting.

Purification and characterization of tagged proteins

To purify the principal SsrA-DD-tagged product from cells expressing λN-4AGA, a 3 l culture of strain X90 *ssrA::cat* containing plasmids pKW22 and pER118-1 was grown, harvested, lysed and subjected to denaturing Ni^{2+}-NTA chromatography according to the Qiagen protocol with minor modifications. All lysis, binding and washing steps were carried out using buffer A (6 M guanidine–HCl, 0.1 M NaH_2PO_4, 0.01 M

Tris, pH 8) containing 10 mM imidazole. The λN-4AGA proteins were eluted with buffer A containing 250 mM imidazole, dialyzed against 30 mM NaH_2PO_4, 50 mM NaCl, 1 mM EDTA, pH 6.1 and chromatographed on a 1 ml MonoS column (Pharmacia) using an FPLC and a linear gradient of 35 ml from 240 to 760 mM NaCl in the same phosphate buffer used for dialysis. Fractions were analyzed by Western blotting using anti-DD antibodies, and although multiple tagged species were observed, the major tagged protein represented approximately two-thirds of the total tagged λN-4AGA products. Those fractions containing the major tagged product were pooled and further purified by HPLC using a Delta-Pak 3.9×150 mm C18 column (Waters) with an 80 ml gradient from 30 to 70% acetonitrile in 0.1% trifluoroacetic acid. The full-length λN-4AGA protein was pooled from a different set of the MonoS fractions and was purified to homogeneity by C18 HPLC chromatography. The extent of tagging was estimated by quantifying Coomassie Blue-stained acrylamide gels of the MonoS fractions and the HPLC absorption profile of the C18 chromatography fractions. The purified proteins were analyzed by MALDI/TOF mass spectrometry on a PerSeptive Biosystems Voyager mass spectrometer using sinipinic acid as the matrix and horse heart myoglobin (Sigma) as an internal standard. Ni^{2+}-NTA purification of λN-AGA proteins translated from mRNAs containing different numbers of AGA codons was carried out in a similar manner, as was purification of λN-AGA-3CGA protein.

Northern blotting and RNase protection

Samples for RNA analysis from tagging experiments *in vivo* were removed to ice ~1 h and 45 min after IPTG induction and were immediately pelleted in a microcentrifuge at top speed for 1 min at 4°C. The supernatant was quickly removed and the pellets were frozen at –80°C. Pellets were resuspended in 100 ml of ice-cold TE buffer containing 0.6 mg/ml lysozyme per OD_{600}/ml and 100 ml of this resuspended mixture was processed according to the RNeasy Miniprep Kit (Qiagen) protocol with rapid digestion (2 min) and processing times.

Northern blotting was performed as described in section 4.9 of *Current Protocols in Molecular Biology* (Chanda, 1997) with modifications. Marker and cellular RNAs were electrophoresed on a 2.8% agarose formaldehyde gel, transferred to a Nytran Plus membrane, probed with 10^5 counts/ml of the ^{32}P-labeled oligonucleotide 5′-AAT TGC TTT AAG GCG ACG TGC-3′ complementary to λN-AGA mRNA, and the membrane was washed with moderate stringency at 42°C. After quantification by PhosphorImaging, the membrane was stripped at 68°C and reprobed with a ^{32}P-labeled oligonucleotide 5′-CCG TCC GCC ACT CGT CAG CAA-3′ complementary to 16S RNA.

RNase protection experiments were performed using methods based on those in section 4.7 of *Current Protocols in Molecular Biology* (1997). Primers 5′-CCG AAT TCT AAT ACG ACT CAC TAT AGG GCA AGC TGA TCC CCG GGT CC-3′ and 5′-TAT AAC GCG GCA TTG CTA GCA AAA-3′ were used to PCR-amplify plasmids pER157 and pER158 in separate reactions. The resulting templates were used to transcribe body-labeled RNA probes for RNase protection assays. Northern blots and RNase protection assays were scanned and quantified using a 445 SI PhosphorImager (Molecular Dynamics) and ImageQuant Version 5.0 software.

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