IL-4, and 40 pg/ml for IL-10.

28. Mice were infected subcutaneously in one hind footpad with 2×10^6 *L. major* LV 39 promastigotes (MRHO/SU/59/P strain). Lesion development was monitored with a dial gauge caliper (Schnelltaster, Kröplin, Germany) and expressed as lesion size = thickness of the infected footpad minus the uninfected contralateral footpad. For the determination of living parasites [R. G. Titus, M. Marchand, T. Boon, J. A. Louis, *Parasite Immunol.* **7**, 545 (1985)], footpad tissues from infected mice were homogenized, and serial 10-fold dilutions were distributed in wells of microtiter plates containing rabbit blood agar slants. After 10 to 14 days of incubation at 26°C, the wells containing growing promastigotes were identified by microscopic examination. The frequency of living parasites recovered from infected footpads was determined by minimum χ^2 analysis applied to a Poisson distribution.
29. Mice were infected as described in Fig. 2. Sera were collected at different times after infection, and the serum concentration of the indicated Ig isotypes were determined by ELISA according to standard protocols. The detection limit for IgG1 and IgG2a were 0.5 ng/ml (Southern Biotechnology), and the detection limit for IgE was 30 ng/ml (PharMingen). Popliteal LN cells (1×10^7 per milliliter) were stimulated with 4×10^6 live *L. major* promastigotes as antigen in 24-well plates for the determination of antigen-specific cytokine production. Nonstimulated and anti-CD3–stimulated (1 μg/ml) cells were used as controls (*12*).
30. Popliteal LN from individual IL-4 control, mutant, and anti–IL-4 (11B11)–treated mice were removed on days 27 and 59 of infection, flash frozen in liquid nitrogen, and homogenized, and total RNA was extracted in Ultraspec (Biotecx). The RNA was treated with DNase I (Gibco, BRL), and 5 μg was reverse-transcribed with murine Moloney leukemia virus reverse transcriptase and oligo$(dT)_{15}$ primers (Superscript II amplification system, Gibco, BRL). For β_2-microglobulin amplification, 5 ng of cDNA per reaction was used and 60 ng was used to amplify cytokine transcripts. The cycling conditions were 20 s at 94°C, 30 s at 56°C, and 30 s at 72°C for 35 cycles. 1 μCi of [α-^{32}P]deoxycytidine triphosphate was included per reaction. Half of the PCR products were run through a 2% agarose gel. Band intensities were quantitated with a Fujix BioImaging Analyzer BAS100 equipped with MacBase software.
31. We thank F. Fitch, J. Augers, and J. Bluestone for their help, advice, and generosity in letting us use their FACScan; W. E. Paul for the gift of IL-4 mAb 11B11; D. Shire, Sanofi Recherche, France, for primer sequences and plasmid pMus3; K. Geiger for help with the statistics; B. Ledermann for helpful discussions; and R. Etges for his comments on the manuscript. Partially supported by funds from the University of Notre Dame (I.M. and P.K.), NIH grant NIH-1R29 AI37636-01 (I.M.), and the American Cancer Society and United States Public Health Service (B. Knowles). N.N.-T. was supported by a postdoctoral fellowship from the National Cancer Institute.

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Role of a Peptide Tagging System in Degradation of Proteins Synthesized from Damaged Messenger RNA

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Variants of λ repressor and cytochrome b_{562} translated from messenger RNAs without stop codons were modified by carboxyl terminal addition of an *ssrA*-encoded peptide tag and subsequently degraded by carboxyl terminal–specific proteases present in both the cytoplasm and periplasm of *Escherichia coli*. The tag appears to be added to the carboxyl terminus of the nascent polypeptide chain by cotranslational switching of the ribosome from the damaged messenger RNA to *ssrA* RNA.

Although many intracellular proteases have been identified (*1*), the determinants that render certain proteins the targets of particular proteases are generally not known. Studies of recombinant interleukin-6 (IL-6) purified from inclusion bodies of *Escherichia coli* revealed that some IL-6 molecules are modified by COOH-terminal truncation at different positions in the sequence and addition of an 11-residue peptide tag (AANDENYALAA) (*2*) at the truncation point (*3*). The last 10 residues of this peptide tag are encoded by the *ssrA* gene of *E. coli*, and tagging of IL-6 does not occur in *ssrA*$^-$ cells. The *ssrA* transcript is a stable 362-nucleotide RNA molecule that exhibits some tRNA-like properties and can be charged with alanine (*4*). The COOH-terminal residues of the *ssrA*-encoded peptide tag (YALAA) are similar to a COOH-terminal tail sequence (WVAAA) recognized by Tsp (*5*), a periplasmic endoprotease, and by a cytoplasmic protease that also degrades proteins in a tail-dependent manner (*6*). Thus, we reasoned that tagging with the *ssrA*-encoded peptide might target proteins for degradation by COOH-terminal–specific proteases.

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To test this hypothesis, we constructed genes encoding variants of the NH_2-terminal domain (residues 1 to 93) of λ repressor (a cytoplasmic protein) and cytochrome b_{562} (a periplasmic protein) in which the *ssrA* peptide tag sequence (AANDENYALAA) was encoded at the DNA level as a COOH-terminal tail (*7*). Variants of each protein were also constructed with a control tag (AANDENYALDD), because both Tsp and its cytoplasmic counterpart do not cleave proteins with charged residues at the COOH-terminus (*5*, *6*). In pulse-chase experiments (Fig. 1, A and B), the λ repressor and cytochrome b_{562} proteins with the *ssrA* peptide tag were degraded with half-lives of <5 min, whereas proteins with the control tag had half-lives of >1 hour. When the *ssrA* peptide–tagged cytochrome b_{562} protein was expressed in an *E. coli* strain deleted for the *tsp* gene, its half-life increased to >1 hour (Fig. 1C). As expected, degradation of the *ssrA* peptide–tagged λ repressor protein in the cytoplasm was not affected in the *tsp*$^-$ strain (Table 1). Thus, the presence of the *ssrA* peptide tag at the COOH-termini of these proteins resulted in rapid intracellular degradation in both the cytoplasmic and periplasmic compartments of the cell. In the periplasm, this rapid degradation required the presence of Tsp. Incubation of the purified variant cytochrome proteins with Tsp in vitro (*8*) resulted in cleavage of the *ssrA* peptide–tagged variant but not of the control peptide–tagged variant (Fig. 1D), thus supporting a direct role for Tsp in the periplasmic degradation of *ssrA* peptide–tagged substrates in vivo (*9*).

Figure 1E shows a model that addresses the mechanisms by which the *ssrA*-encoded tag sequence may be added to the COOH-terminus of a protein and by which specific proteins in the cell may be chosen for modification by peptide tagging, as well as the

Table 1. Half-lives of the λ repressor and cytochrome b_{562} constructs in *tsp*$^+$ *ssrA*$^+$ (X90), *tsp*$^-$ *ssrA*$^+$ (KS1000), and *tsp*$^+$ *ssrA*$^-$ (X90 *ssrA1::cat*) strains. ND, not determined.

Protein construct	Half-life (min)		
	tsp$^+$ *ssrA*$^+$	*tsp*$^-$ *ssrA*$^+$	*tsp*$^+$ *ssrA*$^-$
λ repressor(1–93)–AANDENYALAA	4	4	ND
λ repressor(1–93)–AANDENYALDD	>60	>60	ND
λ repressor(1–93)–M2-H_6-*trpAt*	2	ND	>60
Cyt b_{562}–AANDENYALAA	4	>60	ND
Cyt b_{562}–AANDENYALDD	>60	>60	ND
Cyt b_{562}-*trpAt*	<0.5	~30	ND
Cyt b_{562}–M2-H_6-*trpAt*	<0.5*	~40	~60

*The half-life is that of the full-length protein; a metastable proteolytic intermediate is produced that is degraded with a half-life of ~15 min.

biological rationale for such a system. Specific mRNAs in the cell may lack stop codons because of premature termination of transcription or nuclease cleavage. Completing translation of such mRNAs is potentially problematic, because the ribosomal factors that normally release the nascent polypeptide require the presence of termination codons (*10*). Thus, the ribosome might stall or idle on reaching the 3′ end of the mRNA. We propose that (i) alanine-charged *ssrA* RNA recognizes a ribosome stalled at the end of an mRNA without a stop codon; (ii) the alanine from the *ssrA* RNA is added to the COOH-terminus of the nascent chain, creating a peptidyl–*ssrA* RNA molecule; (iii) translation by the ribosome switches from the 3′ end of the damaged mRNA to the region of the *ssrA* RNA that encodes the tag sequence (*11*); and (iv) normal termination and release occur at the ochre termination codon that follows the peptide tag region of *ssrA* RNA. According to this model, any protein translated from an mRNA that lacks a termination codon will be modified by peptide tagging. As a result, aberrant polypeptides that might be deleterious to the cell can be recognized and degraded by specialized intracellular proteases.

To test this model, we cloned the *trpA* transcriptional terminator (*trpAt*) upstream of the translation termination codons in the 3′ regions of genes encoding the NH_2-terminal domain of λ repressor and cytochrome b_{562} (*12*–*14*). According to the model, both λ repressor (1–93)–M2-H_6-*trpAt* and cytochrome (cyt) b_{562}–*trpAt* transcripts should terminate at *trpAt*, and the resulting protein should be modified by peptide tagging and then degraded. In cells containing *ssrA* RNA, the λ repressor variant was degraded with a half-life of a few minutes (Fig. 2A and Table 1). In isogenic cells lacking *ssrA*, the protein was both smaller (as expected if peptide tagging did not occur) and degraded more slowly (Fig. 2A) (*15*). The cyt b_{562}–*trpAt* protein also appeared to be rapidly degraded in *ssrA*$^+$ cells; in fact, no protein was observed after a 30-s pulse (Fig. 2B). The same protein was detected and longer lived (*16*) in an otherwise isogenic *tsp*$^-$ strain (Fig. 2B). Thus, as predicted by the model, proteins translated from mRNAs that lack termination codons appeared to be modified by *ssrA*-dependent peptide tagging. The half-lives for these *ssrA* peptide–tagged proteins were short (<2 min) in wild-type strains but were increased markedly (30 to 60 min) for peptide-tagged periplasmic variants in strains lacking Tsp. These observations provide further evidence that *ssrA*-mediated peptide tagging targets proteins for degradation by specialized proteases.

A second cytochrome b_{562} protein (cyt b_{562}–M2-H_6-*trpAt*) in which *trpAt* was separated from the body of the protein by an M2 epitope and His_6 sequence was also rapidly cleaved (half-life, <30 s) in cells containing Tsp, but in this instance cleavage resulted in a metastable intermediate (Fig. 2C). This proteolytic intermediate was

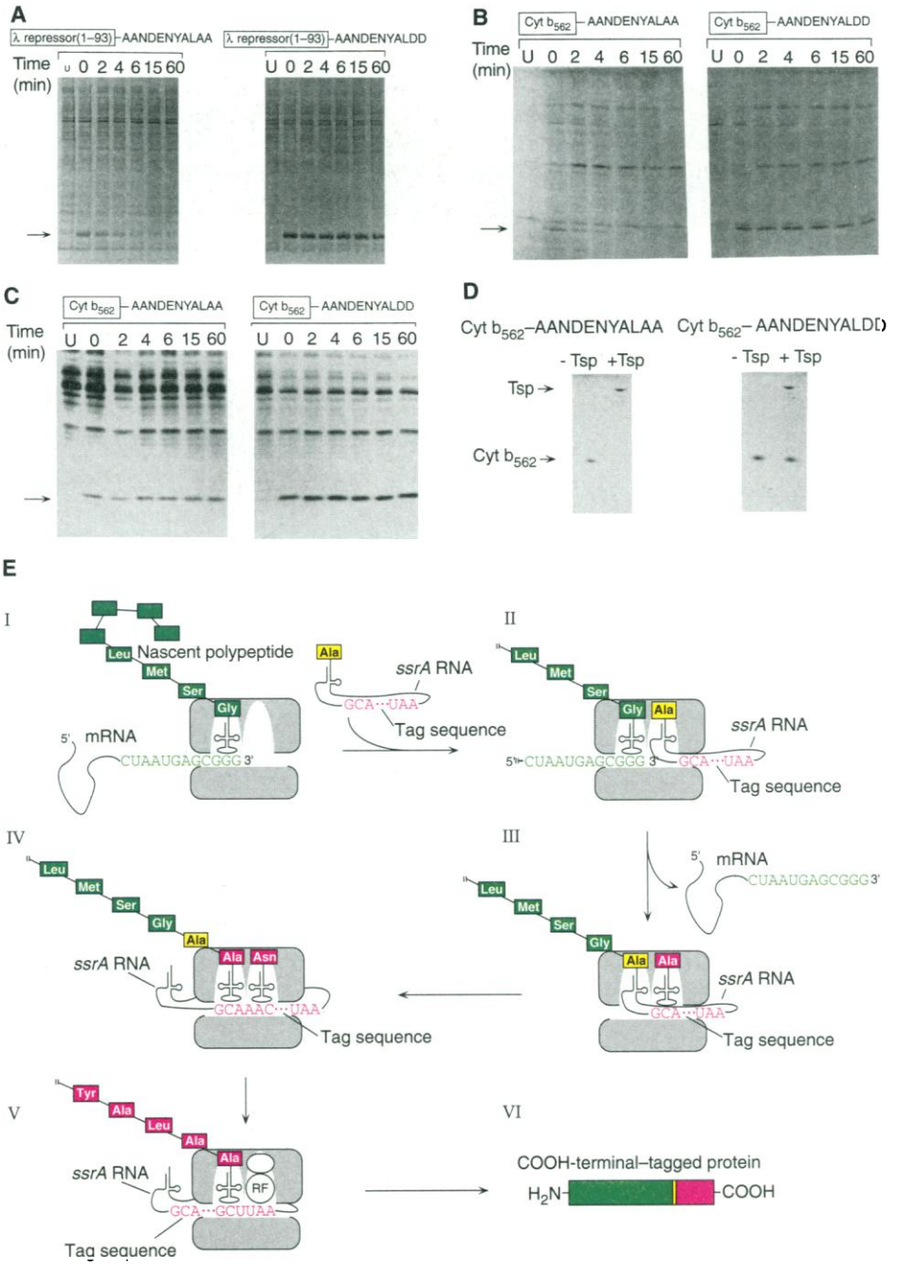

Fig. 1. Degradation of variants of the NH_2-terminal domain of λ repressor [λ repressor (1–93)] or cytochrome b_{562} fused to the *ssrA* peptide tag (AANDENYALAA) or a control tag (AANDENYALDD). (**A**) Pulse-chase assays (*27*) for the λ repressor variants in the *tsp*$^+$ *E. coli* strain X90 (*6*). Arrow indicates the induced protein. Lane U, uninduced control. (**B**) Pulse-chase assays of the cytochrome (Cyt) b_{562} variants in strain X90. (**C**) Pulse-chase assays of the cytochrome b_{562} variants in the *tsp*$^-$ strain KS1000 (*6*). (**D**) Degradation of the purified AANDENYALAA variant of cytochrome b_{562}, but not the AANDENYALDD variant, by Tsp in vitro (*8*). (**E**) Model for *ssrA* RNA–mediated peptide tagging of proteins synthesized from mRNAs without termination codons. (I) The ribosome reaches the 3′ end of the mRNA, leaving the nascent polypeptide esterified to the tRNA in the A site. (II) The stalled complex is recognized by alanine-charged *ssrA* RNA, which binds in the P site. (III) Alanine from the *ssrA* RNA is added to the nascent polypeptide chain, and the ribosome switches translation to the region of *ssrA* RNA that encodes the peptide tag. (IV) The *ssrA* peptide tag is added cotranslationally to the growing nascent chain. (V) The *ssrA* peptide–tagged protein is released from the ribosome when the ochre termination codon of *ssrA* RNA is reached. (VI) The *ssrA* peptide–tagged protein can be recognized and degraded by specialized COOH-terminal–specific intracellular proteases.

then degraded with a half-life of ~15 min (*17*). In cells lacking the Tsp protease, a larger, uncleaved form of the protein was produced (Fig. 2C). The intermediate and uncleaved forms of the cyt b_{562}–M2-H_6-*trpAt* protein were purified from *tsp*$^+$ and *tsp*$^-$ strains, respectively, and characterized by NH_2-terminal sequencing and ion spray mass spectrometry (*18*). The protein purified from the *tsp*$^-$ strain had the NH_2-terminal sequence (ADLED) of cytochrome b_{562} (*19*) but was heterogeneous by mass spectrometry; the largest and most abundant form had a mass of 15,637 daltons, within 0.06% of that expected for a protein containing residues 1 to 129 of the cyt b_{562}–M2-H_6-*trpAt* gene followed by the AANDENYALAA peptide tag (Fig. 3B). The protein purified from the *tsp*$^+$ strain had the same NH_2-terminal sequence and a mass of 13,919 daltons, within 0.05% of that expected for a protein consisting of residues 1 to 123 (Fig. 3B). These data suggest that Tsp initially cleaved the mature protein at an A ↓ A sequence, thereby removing ~15 to 20 residues.

To confirm the peptide-tagged structure proposed above, we digested the cyt b_{562}–M2-H_6-*trpAt* proteins purified from *tsp*$^+$ and *tsp*$^-$ strains with trypsin and separated the products by chromatography on a C_{18} column. Two peptides produced only with the protein from the *tsp*$^-$ strain (labeled 1 and 2 in Fig. 3A) were sequenced by Edman degradation. Peptide 1 contained four residues encoded by the *trpAt* portion of the cyt b_{562}–M2-H_6-*trpAt* gene followed by the AANDENYALAA peptide tag (Fig. 3B). Peptide 2 was a mixture of sequences containing either two or three *trpAt*-encoded residues followed by the 11-residue tag (Fig. 3B). Thus, in both instances the sequences of the peptides corresponded to those expected if translation of the cytochrome b_{562} variant ended within *trpAt* and the nascent polypeptides were then modified by addition of alanine and the *ssrA*-encoded peptide tag sequence (*20*).

Our results show that proteins synthesized from mRNAs that lack a translational termination codon are modified by addition of an AANDENYALAA peptide tag in an *ssrA*-dependent manner (*21*). Tagged proteins are then degraded by specialized proteases in both the periplasm and cytoplasm. The connection between tagging and the absence of termination codons is readily explained by the cotranslational model shown in Fig. 1E. The observations that *ssrA* RNA is associated with ribosomes, has tRNA-like properties, can be charged with alanine, and encodes all but the first alanine of the tag peptide (*3*, *4*) also support a cotranslational model. In addition, several observations make pretranslational RNA splicing or posttranslational peptide-ligation mechanisms unlikely. In studies of IL-6 peptide tagging, mRNAs containing both IL-6 and *ssrA* peptide tag sequences were not detected (*3*). Furthermore, the presence of the first alanine of the peptide tag, which is not encoded by either mRNA or *ssrA* RNA, is also difficult to explain by pre- or posttranslational mechanisms.

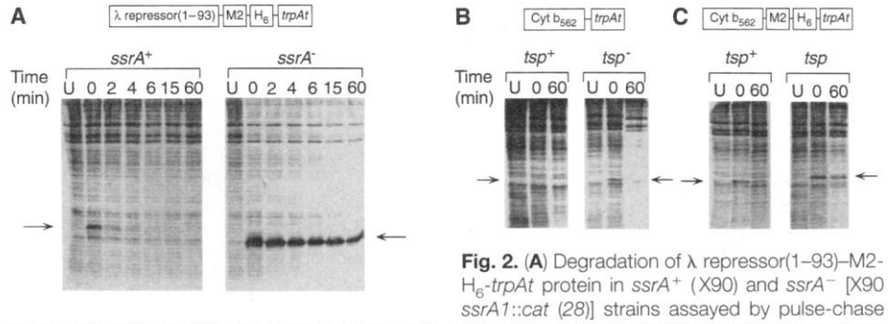

Fig. 2. (**A**) Degradation of λ repressor(1–93)–M2-H_6-*trpAt* protein in *ssrA*$^+$ (X90) and *ssrA*$^-$ [X90 *ssrA1::cat* (*28*)] strains assayed by pulse-chase experiments (*27*). The half-life of the protein is 2 min in the *ssrA*$^+$ strain and >60 min in the *ssrA*$^-$ strain. (**B** and **C**) Pulse-chase experiments for cyt b_{562}–*trpAt* (B) and cyt b_{562}–M2-H_6-*trpAt* (C) in *tsp*$^+$ (X90) and *tsp*$^-$ (KS1000) strains with a 30-s ^{35}S-labeling pulse and incubation for 0 or 60 min with unlabeled methionine. Degradation or processing appears to be sufficiently fast in the *tsp*$^+$ strain that no full-length protein is observed at the zero time point.

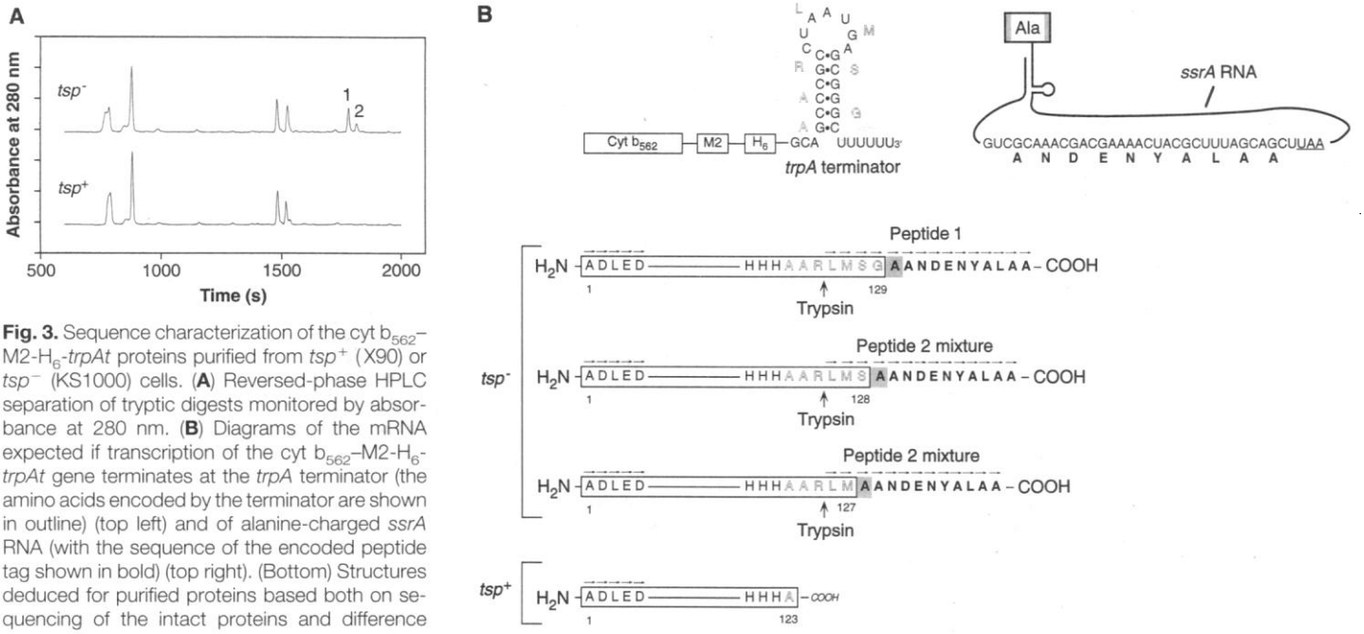

Fig. 3. Sequence characterization of the cyt b_{562}–M2-H_6-*trpAt* proteins purified from *tsp*$^+$ (X90) or *tsp*$^-$ (KS1000) cells. (**A**) Reversed-phase HPLC separation of tryptic digests monitored by absorbance at 280 nm. (**B**) Diagrams of the mRNA expected if transcription of the cyt b_{562}–M2-H_6-*trpAt* gene terminates at the *trpA* terminator (the amino acids encoded by the terminator are shown in outline) (top left) and of alanine-charged *ssrA* RNA (with the sequence of the encoded peptide tag shown in bold) (top right). (Bottom) Structures deduced for purified proteins based both on sequencing of the intact proteins and difference tryptic peptides (arrow indicates position of trysin cleavage) [peaks 1 and 2 in (A)], and on the masses determined by ion spray mass spectrometry. Residues encoded by the cytochrome b_{562}, M2, His_6, and *trpAt* portions of the gene are boxed. Residues encoded by *trpAt* are shown in outline. The alanine esterified to the 3′ end of *ssrA* RNA is shaded. Residues from the peptide tag coding region of *ssrA* RNA are shown in bold. Sequences determined by sequential Edman degradation are indicated by arrows.

The *ssrA* peptide tagging system and associated COOH-terminal–specific proteases probably serve to rid cells of deleterious proteins synthesized from incomplete or otherwise damaged mRNAs. The slow growth of *ssrA*$^-$ strains may reflect the inefficient degradation of damaged proteins (*4*, *22*). Systems involving peptide tagging and degradation by COOH-terminal–specific proteases are likely to be widespread among bacteria, because homologs of *ssrA* and Tsp have been detected in widely varying Gram-negative and Gram-positive species (*4*, *23*).

Obvious parallels exist between the peptide tagging system described here and the ubiquitin system of eukaryotes, which also tags substrates and targets them for degradation (*24*). In addition, some substrates of the N-end rule pathway are modified by addition of specific amino acids in a tRNA-dependent manner (*25*). However, both of these modifications are posttranslational. It remains to be determined whether eukaryotic cells also make use of independent cotranslational peptide tagging mechanisms, similar to those in bacteria, for eliminating proteins synthesized from damaged mRNAs, or whether the spatial separation of transcription and translation in eukaryotic cells allows damaged mRNA to be recognized and degraded before it leaves the nucleus.

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7. Plasmids encoding variants of cytochrome b_{562} with a COOH-terminal AANDENYALAA peptide tag (pCytbSPT) or an AANDENYALDD control tag (pCytbCPT) were constructed by ligating oligonucleotide cassettes encoding the COOH-terminal residues of cytochrome b_{562} followed by the appropriate peptide tail into pNS207, which directs overexpression of wild-type cytochrome b_{562} (*19*).
8. The *ssrA* peptide–tagged and control peptide–tagged variants of cytochrome b_{562} were purified from isopropyl-β-D-thiogalactopyranoside (IPTG)–induced log-phase cultures of the *tsp*$^-$ *E. coli* strains KS1000/pCytbSPT and KS1000/pCytbCPT, respectively (*6*, *7*). Cells were lysed with chloroform, the lysate was acidified to pH 4, insoluble material was removed, and the proteins were purified by chromatography on CM-Acell (Pharmacia), Superdex 75 (Pharmacia), and reversed-phase C_{18} Waters high-performance liquid chromatography (HPLC) columns. The final material was >95% pure as judged by SDS–polyacrylamide gel electrophoresis (PAGE). Cleavage by Tsp in vitro was assayed by incubating 5 μM purified cytochrome b_{562} variant with 300 nM purified Tsp (*5*) at 37°C for 30 min, and analyzing the reaction products by SDS-PAGE with tris-tricine.
9. Appropriate COOH-terminal peptide tails appear to be recognized by and bound to a tethering site on Tsp or its cytoplasmic counterpart, with subsequent cleavage of the tethered substrate mediated by a separate protease active site (*5*, *23*). Substrate cleavage by Tsp does not require adenosine triphosphate or other high-energy compounds, and thus any unfolding of the substrate required before cleavage must occur spontaneously or as a consequence of enzyme binding. The tails themselves are unstructured and do not result in unfolding or decreased thermodynamic stability of, or conformational changes in, the attached protein (*5*, *6*).
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11. Precedents exist for the skipping of as many as 50 nucleotides during translation of the T4 gene 60 mRNA [W. M. Huang *et al.*, *Science* **239**, 1005 (1988)]. This type of skipping from one region of an mRNA to another may be analogous to the ribosome switching proposed here between the 3′ end of the mRNA and the tag sequence of the *ssrA* RNA.
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15. The fact that substantial amounts of stable protein were observed in *ssrA*$^-$ strains for both the λ repressor(1–93)-M2-H_6-*trpAt* and cyt b_{562}-M2-H_6-*trpAt* constructs suggests the existence of a mechanism that allows *ssrA*-independent release of the nascent chain from mRNAs without termination codons. This mechanism may not operate in *ssrA*$^+$ strains or may occur at a slower rate than *ssrA*-mediated peptide tagging and release. The residual slow degradation of these λ and cytochrome variants apparent in *ssrA*$^-$ strains may be attributable to any of several intracellular proteases.
16. The *ssrA* peptide–tagged forms of cyt b_{562}–*trpAt* and cyt b_{562}–M2-H_6-*trpAt* have half-lives of 30 to 40 min in *tsp*$^-$ strains and the cyt b_{562}–AANDENYALAA protein has a half-life of slightly more than 1 hour. Periplasmic proteases homologous to DegP can substitute for Tsp under some conditions [S. Bass, Q. Gu, A. Christen, *J. Bacteriol* **178**, 1154 (1996)] and may be responsible for the relatively slow, Tsp-independent degradation observed for tagged variants in the periplasm. The small differences in the half-lives of these variants in *tsp*$^-$ strains suggest that the position of the peptide tag or the presence of *trpAt* sequences may affect susceptibility to such proteases.
17. The processed form of the cyt b_{562}-M2-H_6-*trpAt* protein may also be degraded by Tsp because its COOH-terminal alanine is the residue most preferred by this protease (K. C. Keiler and R. T. Sauer, *J. Biol. Chem.* **271**, 2589 (1996).
18. The cyt b_{562}-M2-H_6-*trpAt* proteins were purified from IPTG-induced cultures of *E. coli* strain X90 (*tsp*$^+$) or KS1000 (*tsp*$^-$) that had been transformed with appropriate plasmids. Cells were lysed in 6 M guanidine-HCl by sonication, the insoluble fraction was removed by centrifugation, and the supernatant was applied to a 5-ml Ni–nitrilotriacetic acid agarose column. Bound proteins were eluted in buffer containing 250 mM imidazole and separated by chromatography on a C_{18} reversed-phase HPLC column. Fractions containing cyt b_{562}-M2-H_6-*trpAt* of >95% purity by SDS-PAGE were pooled, dried under vacuum, and resuspended in 10 mM tris (pH 8.0) and 20 mM KCl. Approximately 5 μg of purified protein were incubated with 0.1 μg of TPCK (tosylphenylalanyl-chloromethyl ketone)–trypsin at 37°C for 30 min, and the products were separated on a C_{18} reversed-phase HPLC column with a water and acetonitrile gradient in 0.1% trifluoroacetic acid. Amino-terminal sequencing was performed by sequential Edman degradation, and masses were determined by electrospray mass spectrometry.
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20. As shown in Fig. 3B, at least three different peptide-tagged forms of the cyt b_{562}-M2-H_6-*trpAt* protein were purified from the *tsp*$^-$ strain. These differ in having five, six, or seven *trpA*-encoded residues. Similarly, numerous different peptide-tagged forms of IL-6 were identified by Tu *et al.* (*3*). We assume that these tagged IL-6 proteins were not degraded because they form inclusion bodies. Size heterogeneity of the IL-6 mRNA was also observed (*3*). By our model, mRNA heterogeneity naturally results in heterogeneity of the tagged protein products. This mRNA heterogeneity could arise from heterogeneity in transcriptional termination or by nuclease cleavage of full-length mRNA (*13*) [E. Hajnsdorf, O. Steier, L. Coscoy, L. Teysset, P. Regnier, *EMBO J.* **13**, 3368 (1994); H. Causton, B. Py, R. S. McLaren, C. F. Higgins, *Mol. Microbiol.* **14**, 731 (1994)].
21. The simplest model is that tagging occurs when an mRNA lacks a translational termination codon. However, the results do not exclude a possible role for the *trpAt* sequence.
22. In addition to slow growth, *ssrA*$^-$ mutants have phenotypes that reflect changes in intracellular proteolysis (*26*) and alterations in the lysis-lysogeny decisions of temperature bacteriophages [D. M. Retallack, L. J. Johnson, D. I. Friedman, *J. Bacteriol.* **176**, 2082 (1994)]. It is unclear whether these phenotypes arise directly or indirectly from loss of the peptide tagging activity of *ssrA* RNA, with subsequent changes in targeted degradation, or are caused by other defects associated with loss of *ssrA* RNA [D. M. Retallack and D. I. Friedman, *Cell* **83**, 227 (1995)].
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27. For pulse-chase experiments, cells were grown to mid–log phase at 37°C in M9 minimal medium (no Met or Cys). Protein expression was induced by addition of 1 mM IPTG, and after 20 min a labeling pulse of 100 μCi of [^{35}S]methionine was added for 30 s (Figs. 1, A and B, and 2) or 120 s (Fig. 1A). L-Methionine was then added to a final concentration of 1.4 mg/ml. At various times, 0.5-ml portions were removed and the cells were either immediately lysed by boiling in SDS sample buffer (Figs. 1A and 2) or frozen in an ethanol–dry ice bath (Fig. 1, B and C) and lysed by three cycles of thawing at 4°C followed by refreezing. Samples were subjected to SDS-PAGE and ^{35}S-labeled proteins were detected by autoradiography or analysis with a PhosphorImager (Molecular Dynamics). All half-lives were calculated from results for a minimum of six time points by nonlinear least-squares fitting of data to an exponential decay. For these calculations, the intensities of the induced bands were integrated with PhosphorImager ImageQuant software and were normalized to a set of stable bands.
28. Strain X90 *ssrA1::cat* was constructed by P1 transduction of the *ssrA1::cat* allele from *E. coli* strain JK6257 (*26*) into *E. coli* strain X90.

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