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Analysis of the Role of *trans*-Translation in the Requirement of tmRNA for λ*imm*^{P22} Growth in *Escherichia coli*

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The small, stable RNA molecule encoded by *ssrA*, known as tmRNA or 10Sa RNA, is required for the growth of certain hybrid λ*imm*^{P22} phages in *Escherichia coli*. tmRNA has been shown to tag partially synthesized proteins for degradation in vivo by attaching a short peptide sequence, encoded by tmRNA, to the carboxyl termini of these proteins. This tag sequence contains, at its C terminus, an amino acid sequence that is recognized by cellular proteases and leads to degradation of tagged proteins. A model describing this function of tmRNA, the *trans*-translation model (K. C. Keiler, P. R. Waller, and R. T. Sauer, Science 271:990–993, 1996), proposes that tmRNA acts first as a tRNA and then as a mRNA, resulting in release of the original mRNA template from the ribosome and translocation of the nascent peptide to tmRNA. Previous work from this laboratory suggested that tmRNA may also interact specifically with DNA-binding proteins, modulating their activity. However, more recent results indicate that interactions between tmRNA and DNA-binding proteins are likely nonspecific. In light of this new information, we examine the effects on λ*imm*^{P22} growth of mutations eliminating activities postulated to be important for two different steps in the *trans*-translation model, alanine charging of tmRNA and degradation of tagged proteins. This mutational analysis suggests that, while charging of tmRNA with alanine is essential for λ*imm*^{P22} growth in *E. coli*, degradation of proteins tagged by tmRNA is required only to achieve optimal levels of phage growth. Based on these results, we propose that *trans*-translation may have two roles, the primary role being the release of stalled ribosomes from their mRNA template and the secondary role being the tagging of truncated proteins for degradation.

The lambdoid family of bacteriophages has been used extensively as tools in the study of bacterial physiology in *Escherichia coli* (6). Host functions first identified by mutations that affect the growth of lambdoid phages have often later been found to have important roles in physiological processes of the host cell itself. A small, stable RNA, tmRNA (previously known as 10Sa RNA), encoded by the *ssrA* locus of *E. coli*, has been shown to be required for the growth of certain hybrid λ*imm*^{P22} phages (31, 38). *E. coli* tmRNA is present at approximately 1,000 copies per haploid genome (23) and is processed at both ends to create a 363-nucleotide mature form from a 457-nucleotide precursor (21, 24, 35, 36, 39). Homologs of tmRNA have been located in every bacterial species whose genome has been searched (4, 12, 29, 42, 43, 45). Analysis of the *E. coli* tmRNA indicates that this RNA has a secondary structure resembling half of a tRNA molecule (5, 21, 45), including the acceptor stem and TΨC stem-loop of alanyl tRNA in *E. coli*, and in vitro studies show that it can be charged with alanine by using purified components (21). Phenotypes exhibited by *E. coli ssrA* mutants, in addition to effects on the growth of certain λ*imm*^{P22} hybrids, include a slowed growth rate (28), delayed recovery from carbon starvation, reduced motility in soft agar (21), reduced expression of some genes (30), and increased expression of Alp protease (19). Additionally, when an *ssrA* mutation is present in the same cell as a mutation in the *prs* gene, encoding phosphoribosyl pyrophosphate synthetase, an enzyme involved in nucleotide synthesis, bacteria are unable to grow at 42°C (1).

In the last few years, evidence has accumulated supporting a model in which tmRNA tags partially synthesized proteins for degradation by cellular proteases (18, 41). This "*trans*-translation" model, first proposed by Keiler et al. (18), postulates that alanine-charged tmRNA enters ribosomes that have stalled at the 3′ end of a mRNA without having reached a stop codon. The tmRNA acts first as a tRNA, and its alanine is added to the nascent polypeptide. However, after peptide bond formation, the original mRNA is released and tmRNA becomes the template for translation, resulting in the addition of an 11-amino-acid tag sequence to the C terminus of the nascent polypeptide, the last 10 amino acids of which are encoded by tmRNA. The tag sequence contains a protease recognition site at its C terminus, so that tagged proteins become substrates for the proteases Clp, FtsH, and Tsp (10, 13, 18). Recently published work supporting this model has shown that tmRNA is associated with 70S ribosomes but not 30S or 50S subunits or polysomes (20, 40), that charging of tmRNA with alanine is required for association with ribosomes (40), and that translation of polyuridine in vitro, in the presence of tmRNA, produces polyphenylalanine, followed by the tmRNA tag (15).

The effects of *ssrA* mutation on λ*imm*^{P22} growth were first described by Strauch et al. for a strain of *E. coli* with an uncharacterized mutation in *ssrA* called *sip* (38). The *sip* mutation was later shown to result from the excision of a cryptic prophage adjacent to *ssrA*, resulting in alteration of the 3′ end of tmRNA (19, 31). Certain hybrid λ*imm*^{P22} phages, formed by crosses between coliphage λ and its relative from *Salmonella*, phage P22 (8, 33, 49), are unable to grow on *E. coli* carrying this altered form of *ssrA* (38). These same studies established that mutation of P22 *c*1 removes the requirement for tmRNA in λ*imm*^{P22} phage growth (31, 38). P22 C1 protein activates transcription that is responsible for the establishment of repressor synthesis in phage P22 by binding to the −35 region of the P_{RE} promoter (48). P_{RE} is located downstream of the early

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TABLE 1. Bacterial strains and bacteriophages

Strain or phage	Parent strain	Plasmid	Relevant genotype	Source
K37			*galK* Str^r	This laboratory
K8619	K37		*ssrA::cat*	This work
K8664	K8619		$ssrA^+$ Amp^r (At λ*att*)	This work
K8637	K8619		$ssrA^O$ Amp^r (At λ*att*)	This work
K8666	K8619		$ssrA^{DD}$ Amp^r (At λ*att*)	This work
K8668	K8619		Amp^r (At λ*att*)	This work
K8661	K8619	pJW28	$ssrA^+$ Amp^r	This work
K8810	K8619	pJW31	$ssrA^{GA}$ Amp^r	This work
K8812	K8619	pJW32	$ssrA^{GC}$ Amp^r	This work
K8814	K8619	pJW34	$ssrA^{UG}$ Amp^r	This work
K8895	K8619	pJW33	$ssrA^{CG}$ Amp^r	This work
K8465	K37		*clpP::kan*	This work
K8857	K8619		*clpP::kan ssrA::cat*	This work
K5210	N953		*lac galE* $galK_{am}$ trp_{am} ara_{am} $T6^r$ Su^-	This laboratory
K9281	K8637		$ssrA^O$ *clpP::kan*	This work
K9282	K8666		$ssrA^{DD}$ *clpP::kan*	This work
ZH1141	C600		*gal490 bioA mal* λ*c*I857	D. Court
λBDC531			λimm^{21} *lacZ'::supF::'bla*	D. Court
λimm^{P22}*hy25*			λimm^{P22} $c2^5$ *spi*	S. Hilliker
λimm^{P22}*dis*			λimm^{P22} $c2^5$ *dis*	This laboratory

promoter, P_R, and in the opposite orientation. The P_R operon includes genes, *18* and *12*, encoding the phage DNA replication functions and located downstream of P_{RE}. It was proposed that tmRNA interacts directly with the P22 C1 protein, reducing its binding at P_{RE} (30). According to this model, in the absence of tmRNA, the increased occupancy of P22 C1 at P_{RE} would lead to decreased expression of phage genes and a defect in phage growth. Consistent with this model, an interaction was observed between tmRNA and DNA-binding proteins in vitro in gel shift experiments (30). However, more recent work indicates that the interaction between tmRNA and P22 C1 protein in vitro is less specific than that observed previously and that any interaction between tmRNA and P22 C1 is probably nonspecific (45a).

In light of these findings, we have explored the possibility that the *trans*-translation model may provide an explanation for the effects of *ssrA* mutation on λimm^{P22} growth in *E. coli*. To facilitate our analysis, we have divided the *trans*-translation model into four basic steps: first, charging of tmRNA with alanine; second, transfer of the alanine to the nascent peptide and release of the original mRNA; third, translation of the tmRNA tag and dissociation of the translational ternary complex upon reaching the tmRNA-encoded stop codon; and fourth, degradation of tagged proteins by cellular proteases. We present here the results of experiments showing the effects on λimm^{P22} growth of *ssrA* mutations affecting steps 4 and 1 of *trans*-translation. First, we have made or obtained mutations that change the tag sequence itself, rendering tagged proteins resistant to protease degradation, thus affecting step 4 of *trans*-translation; and second, we have made mutations that prevent tmRNA from being charged with alanine, affecting step 1. We have also examined the effects that mutation of *clpP*, the catalytic subunit of the Clp protease (26), has on λimm^{P22} growth. Our results suggest that charging of tmRNA with alanine is critical for λimm^{P22} growth in *E. coli* but that degradation of tagged proteins is required only to achieve an optimal level of phage growth.

MATERIALS AND METHODS

Strains. Bacterial strains used in this study are listed in Table 1, and plasmid genotypes are included where relevant. The genetic organization of the relevant regions of the bacteriophages used is shown in Fig. 1.

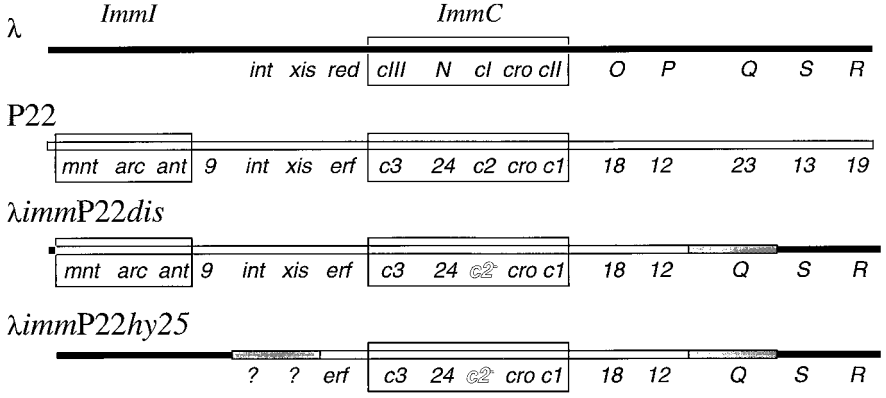

FIG. 1. Genetic organization of the relevant regions of phages λ and P22 and hybrid phages. Immunity regions are boxed. The solid line indicates λ genetic information, and the open line indicates P22 genetic information. The gray lines indicate areas of the phage chromosomes the origins of which have not been determined. Both hybrids used in this study have a mutation that results in production of an inactive repressor protein, C2, shown by outlined lettering.

Media. Bacterial cultures were grown in Luria-Bertani broth, described previously (7).

Cloning procedures. Standard cloning techniques were used (32). Enzymes were purchased from New England Biolabs, Boehringer Mannheim, and Gibco BRL and were used according to the suppliers' instructions.

Construction of plasmids carrying *ssrA* mutants. *ssrA* mutant alleles were constructed by the PCR splicing by overlap extension method (16) and cloned into plasmid pRS415 (34). Mutant sequences were verified by DNA sequencing with the Thermosequenase kit (Amersham).

Construction of single-copy *ssrA* mutants. Single-copy constructs of *ssrA* tag mutants were constructed by the method of Yu and Court (50). Briefly, phage λBDC531 (*imm*^{21}) was grown on a strain carrying an allele of *ssrA* cloned into plasmid pRS415 (34). The resulting phages were used to make lysogens in strain K5210, and the lysogens were selected for ampicillin resistance. Bacteriophage P1 (37) was grown on these lysogens and used to transduce Amp^r into strain ZH1141 made resistant to λ. P1 was then grown on this strain, and Amp^r was transduced into K8619. The final constructs are sensitive to phages λ and 21 and are resistant to ampicillin and chloramphenicol (*ssrA::cat*).

EOP. The efficiency of plating (EOP) of phages was determined as described previously (2).

Bursts of phages. Phage bursts were measured as described previously (38). Experiments were performed at 37°C for 90 min. Each value given is the average (± the standard deviation) of at least three separate experiments.

Northern blot analysis. RNA was purified from late-logarithmically growing cultures of the specified strain with the RNeasy total RNA kit (Qiagen). The purified RNA was electrophoresed on a 2% agarose gel containing 20% formaldehyde in 1× formaldehyde-gel running buffer (20 mM MOPS [morpholinepropanesulfonic acid], pH 7.0, 8 mM sodium acetate, 5 mM EDTA, pH 8.0). The RNA was transferred to a GeneScreen *Plus* hybridization transfer membrane (NEN) by electrotransfer on a SemiPhor apparatus (Hoefer). End-labeled oligonucleotide probes specific for tmRNA or 16S rRNA were hybridized to the immobilized RNA, and the blots were washed as described by Sambrook et al. (32). The hybridized probe was visualized and quantitated with a PhosphorImager system (Molecular Dynamics).

RESULTS

Description of phages and effects of *ssrA::cat* mutation on λ*imm*^{P22} growth. Two different λ*imm*^{P22} hybrid phages were used to assess the effects of various mutations in *ssrA* on λ*imm*^{P22} growth. The two hybrids described here (Fig. 1) were chosen because of the known effects of *ssrA* mutation upon their growth (31, 38). The first, λ*imm*^{P22}*dis* (49), has a relatively large region from *Salmonella* phage P22 replacing a homologous region from phage λ and carries both of the immunity regions, *immC* and *immI*, from P22. *immC* encodes gene products that determine whether the phage assumes the lysogenic pathway or grows lytically, and it is analogous to the immunity region of phage λ. *immI* regulates the expression of an antirepressor protein, Ant, and an analogous region is not found in λ (14). λ*imm*^{P22}*dis* also carries the integration and excision genes, recombination function, and DNA replication genes from P22. The remainder of the phage genome is derived from λ. The second hybrid, λ*imm*^{P22}*hy25* (14), has a smaller region of the genome from P22 replacing that of λ. This hybrid also carries the DNA replication, recombination, and *immC* regions from P22 but does not carry the second immunity region, *immI*. The derivation of the region of the λ*imm*^{P22}*hy25* genome between the recombination function locus, *erf*, and the λ region replacing *immI* (Fig. 1) has not been determined. The derivation of the transcription factor required for expression of phage late genes, termed Q in phage λ and 23 in phage P22, has not been determined for either hybrid used in this study.

The effects on λ*imm*^{P22} growth of an *ssrA::cat* insertional mutation have been previously described by this and another laboratory (19, 31). However, experiments performed with our existing *ssrA::cat* strain have yielded results that are not entirely consistent, presumably due to acquisition of an additional mutation(s). Therefore, we have constructed a new *ssrA::cat* strain by crossing an *ssrA::cat* allele (19) into K37, our standard *E. coli* strain, creating strain K8619 (Table 1). We tested the ability of the newly constructed *ssrA::cat* strain to support growth of λ*imm*^{P22} hybrid phages, (see Fig. 3, 5, and 7). The EOPs of λ*imm*^{P22}*hy25* and λ*imm*^{P22}*dis* in K8619 are decreased by greater than 10,000-fold compared to the EOPs of these hybrid phages seen in the *ssrA*$^+$ strain. A Northern blot experiment demonstrated, as expected, that the *ssrA::cat* strain, K8619, failed to produce detectable levels of tmRNA, unlike the *ssrA*$^+$ strain, K37 (see Table 3, first two lines).

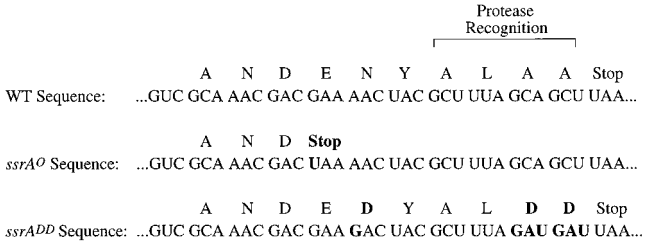

FIG. 2. Sequence of the regions of wild-type and mutant tmRNAs encoding the tag and the presumed amino acid sequence of the resulting tag. Changes from the wild-type sequence are indicated by bold letters. The protease recognition sequence is indicated at the C terminus of the tag.

Testing the role of the degradation step of *trans*-translation in λ*imm*^{P22} growth. To examine whether the *trans*-translation model, as proposed, might explain the defect in λ*imm*^{P22} growth seen in *ssrA* mutant strains, we first tested whether the final step, degradation of tmRNA-tagged proteins, is required for support of λ*imm*^{P22} growth. Derivatives of K8619 (*ssrA::cat*) were constructed that carry a single copy of the wild-type or a mutant allele of *ssrA*. This second *ssrA* allele, including its associated wild-type *ssrA* promoter, was inserted at the λ attachment site. As a control, a derivative of K8619 was constructed in which the cloning vector alone was inserted in single copy at the λ*att* site. Two *ssrA* mutants were studied, each having altered sequences in the region of *ssrA* encoding the tag (Fig. 2). The first, the *ssrA*^{O} mutant, has a single-base-pair change that creates an ochre stop codon in frame early in the tag coding sequence. Inserting a nonsense mutation in the region of tmRNA encoding the tag was the suggestion of T. Silhavy. A tag produced from this allele would lack the hydrophobic protease recognition sequence found at the C terminus of the tag (Fig. 2). The second mutant, the *ssrA*^{DD} mutant, containing an *ssrA* allele obtained from R. Sauer, has two aspartic acid residues replacing the two alanines found at the C terminus of the tag in wild-type *ssrA* as well as an aspartic acid replacing the asparagine earlier in the tag sequence (18). Proteins containing tags from this allele were shown to have an increased half-life in vivo compared to proteins containing a wild-type tmRNA tag, due to disruption of the hydrophobic protease recognition site by the charged aspartic acid residues (18).

The EOPs of two λ*imm*^{P22} phages on these single-copy *ssrA* constructs were measured, and the results are shown in Fig. 3. As noted above, both λ*imm*^{P22}*hy25* and λ*imm*^{P22}*dis* show a greater-than-10,000-fold reduction in EOP on an *ssrA::cat* strain compared to the EOP measured on a strain of *E. coli* with wild-type *ssrA*. When a wild-type copy of *ssrA* is present in single copy in addition to the *ssrA::cat* allele, the EOP returns to wild-type levels. Similarly, when a single copy of either the *ssrA*^{O} or *ssrA*^{DD} allele is present in an *ssrA::cat* background, both λ*imm*^{P22} hybrids show EOPs close to wild-type levels, although the plaques produced by λ*imm*^{P22}*dis* are much smaller on the *ssrA*^{O} and *ssrA*^{DD} strains than on *ssrA*$^+$ *E. coli*. As expected, both phages failed to grow in a derivative of K8619 with the cloning vector integrated at the λ attachment

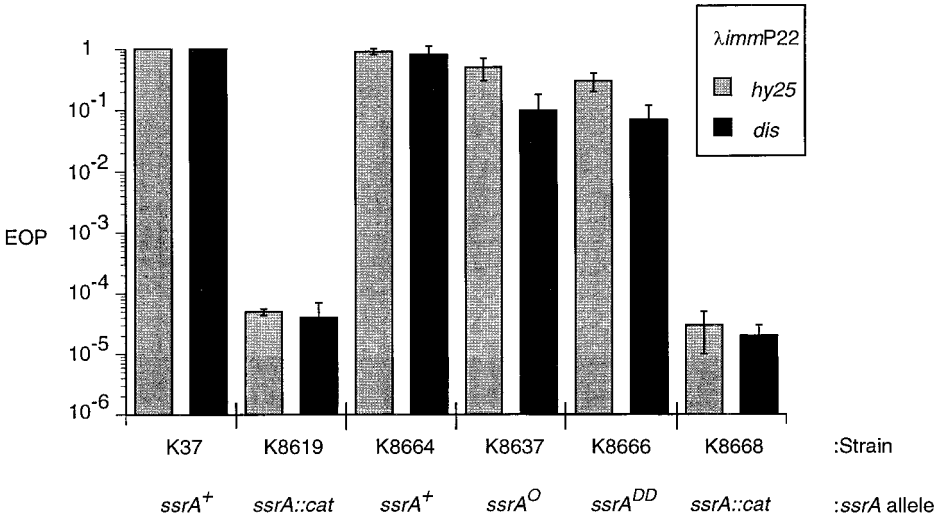

FIG. 3. EOP of λ*imm*^{P22} hybrids on *E. coli* with wild-type or tag mutant *ssrA* alleles. Strains K8664, K8637, and K8666 have the indicated *ssrA* allele inserted at the λ*att* site in addition to the *ssrA::cat* allele at the normal *ssrA* locus. K8668 has the cloning vector alone inserted at λ*att*. The values presented are the averages of three separate experiments, and error bars are included where applicable. The plaques produced by λ*imm*^{P22}*dis* on K8637 and K8666 were tiny relative to plaques produced by λ*imm*^{P22}*dis* on other strains.

site, confirming that it was the *ssrA* alleles that were responsible for growth of the hybrid phages.

Similar experiments were also performed with *ssrA* alleles carried on multicopy plasmids, and we found that the *ssrA*$^+$, *ssrA*^{O}, and *ssrA*^{DD} alleles again supported growth of the hybrid phages while the cloning vector alone did not (data not shown). The small plaque size observed when the EOP of λ*imm*^{P22}*dis* was measured on the *ssrA*^{O} and *ssrA*^{DD} strains suggested that the degradation of tagged proteins could play a role in the growth of λ*imm*^{P22}*dis*. We used burst size as a more quantitative measure of the effectiveness of the *ssrA*^{O} and *ssrA*^{DD} strains in supporting the growth of the hybrid phages (Table 2, lines 1 to 6). The burst sizes of λ*imm*^{P22}*hy25* paralleled the results seen for EOP, i.e., the burst size of λ*imm*^{P22}*hy25* on the *ssrA*^{O} strain was similar to that on the *ssrA*$^+$ strain while the burst of λ*imm*^{P22}*hy25* in the *ssrA*^{DD} strain was slightly lower, ~40% of that measured on the *ssrA*$^+$ strain. Significantly, each of these three *ssrA* alleles supported a burst of λ*imm*^{P22}*hy25* that was 400- to 800-fold higher than that produced on the *ssrA::cat* control strain.

However, the results with λ*imm*^{P22}*dis* in the *ssrA*^{O} and *ssrA*^{DD} strains were quite different. λ*imm*^{P22}*dis* produced bursts that were 34- and 38-fold higher, respectively, than those observed on the *ssrA::cat* control strain but were ~50-fold lower than the burst produced by λ*imm*^{P22}*dis* on an isogenic *ssrA*$^+$ strain. This intermediate phenotype suggests that tmRNA may function in two ways in supporting λ*imm*^{P22} growth. First, as clearly indicated by the results with λ*imm*^{P22}*hy25*, one role of tmRNA is unrelated to the activity that directs peptides toward degradation. Second, as shown by the results with λ*imm*^{P22}*dis*, the activity of tmRNA that directs proteins toward degradation is also required to achieve optimal growth of this hybrid phage.

Testing the role of tmRNA charging in λ*imm*^{P22} growth. The effects of mutations in the tmRNA acceptor stem that affect its ability to be charged with alanine were next examined to determine whether alanine charging, the first step postulated for *trans*-translation, is important for λ*imm*^{P22} growth. Four *ssrA* alleles with altered nucleotide sequences in the acceptor stem were constructed in plasmid pRS415 (Fig. 4). The G · U base pair at the third position in this stem was targeted for mutagenesis because it is the recognition site for charging by the alanyl aminoacyl-tRNA synthetase, so that tmRNA with a change in either of these nucleotides should not be charged with alanine

TABLE 2. Phage burst sizes on *ssrA* and *clpP* mutant strains

No.	Strain	Relevant alleles	Location of relevant *ssrA* allele	λ*imm*^{P22}*hy25* burst	Fold increase over *ssrA::cat*[a]	λ*imm*^{P22}*dis* burst	Fold increase over *ssrA::cat*[a]
1	K37	*ssrA*$^+$ *clpP*$^+$	Normal site	210 ± 36	1,750	160 ± 10	2,500
2	K8619	*ssrA::cat clpP*$^+$	Normal site	0.12 ± 0.08		0.064 ± 0.044	
3	K8664	*ssrA*$^+$ *clpP*$^+$	λ*att*	120 ± 31	1,000	110 ± 43	1,720
4	K8637	*ssrA*^{O} *clpP*$^+$	λ*att*	95 ± 30	790	2.2 ± 1.4	34
5	K8666	*ssrA*^{DD} *clpP*$^+$	λ*att*	50 ± 8.5	420	2.4 ± 1.4	38
6	K8668	*ssrA::cat clpP*$^+$	Normal site	0.078 ± 0.061	0.65	0.021 ± 0.025	0.33
7	K8814	*ssrA*^{UG} *clpP*$^+$	Plasmid	0.098 ± 0.082	0.82	0.036 ± 0.041	0.56
8	K8465	*ssrA*$^+$ *clpP::kan*	Normal site	90 ± 14	750	78 ± 23	1,220
9	K8857	*ssrA::cat clpP::kan*	Normal site	52 ± 33	430	1.2 ± 0.36	19
10	K9281	*ssrA*^{O} *clpP::kan*	λ*att*	ND[b]	ND	94 ± 28	1,470
11	K9282	*ssrA*^{DD} *clpP::kan*	λ*att*	ND	ND	71 ± 29	1,110

[a] The indicated burst sizes were divided by the burst size measured for the *ssrA::cat* strain, K8619.
[b] ND, not determined.

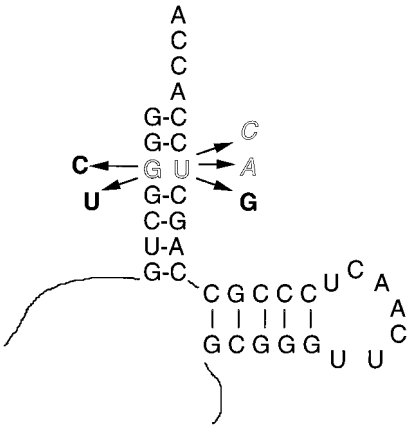

FIG. 4. Acceptor stem and associated sequence of tmRNA showing changes resulting from *ssrA* mutation. The resulting tmRNAs should be defective in charging with alanine. The G · U base pair at the third position of the acceptor stem was changed to G · C or G · A, as shown by outlined letters, or C · G or U · G, as shown by bold letters.

(17, 27). These "charging mutant" alleles cloned in pRS415 were transformed into the *ssrA::cat* strain, K8619, and growth of λ*imm*^{P22} hybrids was measured by EOP (Fig. 5). K8619 carrying an *ssrA*$^{+}$ allele in pRS415 supported growth of both hybrid phages tested, as expected. However, derivatives of K8619 carrying *ssrA* alleles with changes in the G · U base pair to G · A, G · C, C · G, or U · G, respectively, in pRS415 were all unable to support the growth of either hybrid phage tested. It is noteworthy that the final mutation, G · U to U · G, creates an altered sequence, preventing charging of tmRNA with alanine, but does not alter the base-pairing energy and thus should not produce significant differences in structure compared to the wild-type tmRNA, although stacking of bases may be affected by the change. As shown in Fig. 5, this mutant was unable to support λ*imm*^{P22} growth.

To assess more quantitatively the effects of the *ssrA* alleles defective in alanine charging on phage growth, we measured phage burst size in a strain carrying *ssrA*^{UG} as the only intact *ssrA* allele. As shown in line 7 of Table 2, both λ*imm*^{P22} phages tested on a strain carrying the *ssrA*^{UG} allele had burst sizes that were essentially identical to that produced in the *ssrA::cat* control strain.

TABLE 3. Relative levels of tmRNA produced in *ssrA* mutant strains

Strain	*ssrA* allele	Location of relevant *ssrA* allele	Relative tmRNA level[a]
K37	*ssrA*$^{+}$	Normal site	1.0
K8619	*ssrA::cat*	Normal site	ND[b]
K8664	*ssrA*$^{+}$	λ*att*	7.5
K8637	*ssrA*^{O}	λ*att*	1.4
K8666	*ssrA*^{DD}	λ*att*	0.5
K8668	*ssrA::cat*	Normal site	ND
K8810	*ssrA*^{GA}	Plasmid	12.5
K8812	*ssrA*^{GC}	Plasmid	1.9
K8814	*ssrA*^{UG}	Plasmid	6.4
K8895	*ssrA*^{CG}	Plasmid	6.1

[a] The ratio of tmRNA to 16S rRNA measured for the wild-type strain, K37, was set equal to 1, and the tmRNA/16S rRNA ratios measured in the other strains were normalized to this value.
[b] ND, not detectable.

Northern analysis of *ssrA* mutants. To determine whether mutant tmRNAs were being stably expressed, Northern blot analysis was performed, and the results are shown in Table 3. Each of the strains carrying a mutant *ssrA* allele produced an RNA species of a size equal to that seen in a strain carrying a wild-type *ssrA* allele when probed with an oligonucleotide specific for *ssrA* (not shown). A tmRNA signal was not observed in the RNA preparations isolated from strains carrying only the *ssrA::cat* allele, K8619 and K8668. To control for the amount of RNA loaded into each lane of the gel, the blots were simultaneously probed with an oligonucleotide specific for the 16S rRNA. The ratio of tmRNA to 16S rRNA was calculated by quantitating the signal detected in each lane for the tmRNA and 16S rRNA probes with a PhosphorImager and then dividing the amount of signal detected for tmRNA by the amount detected for 16S rRNA. This number was assigned a value of 1 for the wild-type *ssrA* strain, K37, while the ratios measured for the other strains were normalized to this value. A representative experiment is shown in Table 3.

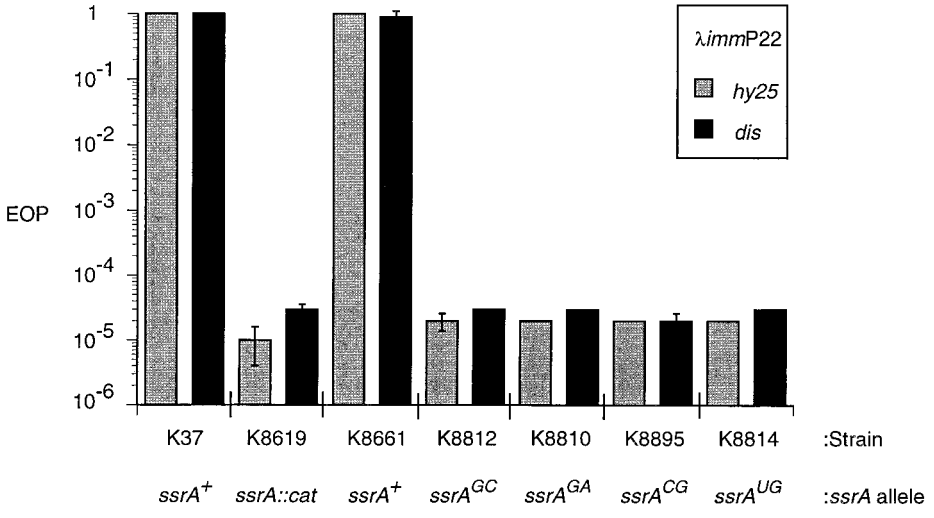

FIG. 5. EOP of λ*imm*^{P22} hybrids on *E. coli* with wild-type or charging mutant *ssrA* alleles. Strains K8661, K8812, K8810, K8895, and K8814 carry the indicated *ssrA* allele cloned in plasmid pRS415. The values presented are the averages of three separate experiments, and error bars are included where applicable.

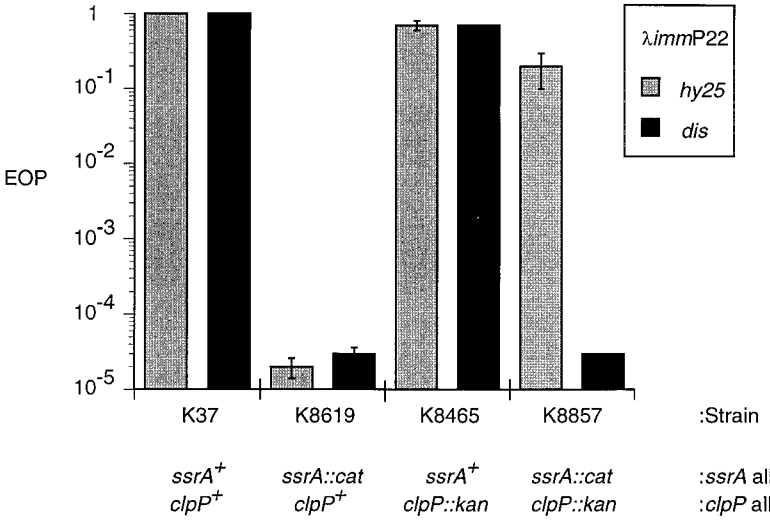

FIG. 6. EOP of λ*imm*^{P22} hybrids on *E. coli ssrA* and *clpP* mutant strains. The values presented are the averages of three separate experiments, and error bars are included where applicable. The plaques produced by λ*imm*^{P22}*hy25* on K8857 were tiny relative to plaques produced by λ*imm*^{P22}*hy25* on other strains.

The ratio of signals shown in Table 3 suggests that each of the *ssrA* mutant strains produces stable tmRNA while the two strains lacking a functional *ssrA* allele do not. While there is some variation among strains, most of the mutant strains produced normalized tmRNA levels at or above the wild-type level of 1. As might be expected, strains K8810, K8812, K8814, and K8895, which carry mutant *ssrA* alleles on multicopy plasmids, exhibited tmRNA levels severalfold higher than that seen in the wild-type strain, K37, which carries a single copy of *ssrA*. The two strains carrying single copies of *ssrA* alleles with altered tag sequences, K8637 and K8666, exhibited tmRNA levels that were quite similar to that seen for the wild-type *ssrA* strain, K37, although the *ssrA*^{DD} strain, K8666, often produced a tmRNA level slightly lower than that seen in K37. The control strain K8664, carrying what we presume by its construction to be a single copy of wild-type *ssrA* at λ*att*, consistently exhibited a tmRNA level higher than that seen in K37, the strain carrying wild-type *ssrA* at its normal location. However, as seen in the EOP and burst experiments performed with these two strains, there was little or no difference in phage growth due to the difference in tmRNA levels between these two control strains.

These results provide compelling evidence that both the derivatives of K8619 carrying copies of *ssrA* with mutations changing the G · U base pair in the acceptor stem and the derivatives of K8619 carrying single copies of *ssrA* with altered tag-coding sequences produce stable tmRNA at levels sufficient for a functional tmRNA to support phage growth.

Effect of a *clpP* mutation on growth of λ*imm*^{P22}. If degradation of tagged proteins is important for growth of the hybrid phages, then we would expect that the λ*imm*^{P22} hybrids would also be inhibited if the responsible protease is not active. The Clp protease (11, 44) has been shown to be the major protease responsible for degradation of tagged proteins in vivo; tagged proteins exhibit an increased half-life in a *clpP* mutant strain of *E. coli* (10, 13). We introduced a *clpP::kan* allele, the gift of S. Gottesman, into our strain background and assayed its effects upon the growth of λ*imm*^{P22} hybrids. As shown in Fig. 6, neither of the λ*imm*^{P22} hybrids tested showed a significant difference in EOP on lawns formed with the *clpP::kan* or *clpP*$^{+}$ strains.

To obtain a more quantitative assessment, we measured the burst sizes of the λ*imm*^{P22} phages in the *clpP::kan* strain. Although EOP measurements failed to detect any significant difference between the wild-type and *clpP::kan* strains in support of the growth of these phages, measurement of burst sizes revealed a small but reproducible difference in support of the growth of both λ*imm*^{P22}*hy25* and λ*imm*^{P22}*dis*; the burst in the *clpP::kan* strain is reproducibly twofold lower than that found in the isogenic strain that has a wild-type *clpP* locus. These twofold differences are relatively insignificant, however, when compared to the 750-fold (λ*imm*^{P22}*hy25*) or 1,220-fold (λ*imm*^{P22}*dis*) differences exhibited when bursts produced in *clpP::kan* and *ssrA::cat* strains are compared.

The *clpP::kan* allele was also introduced into K8619, our *ssrA::cat* strain, to determine whether the absence of the Clp protease could have an effect on phage growth in an *ssrA::cat* background. Given the results described above, which suggest that the charged form of tmRNA is required for λ*imm*^{P22} growth, we hypothesized that some aspect of translation could play a role in the action of tmRNA in supporting hybrid-phage growth. One plausible activity could be an influence on the levels of proteins important for the growth of λ*imm*^{P22}. For example, the level of a limiting protein that is protease sensitive could be influenced by tmRNA. If this protein is degraded by the Clp protease, then we would expect that higher levels of this protein would be present in a *clpP::kan* bacterium and, thus, the effects of the *ssrA::cat* mutation on the growth of the hybrid phages could be suppressed in a strain that has both *ssrA::cat* and *clpP::kan* mutations. When the growth of λ*imm*^{P22} was assessed in the *clpP::kan-ssrA::cat* double-mutant strain, K8857, we observed different results with the two λ*imm*^{P22} hybrids (Fig. 6). Consistent with the outlined hypothesis, λ*imm*^{P22}*hy25* has an EOP of close to 1 on a *clpP::kan-ssrA::cat* double mutant, although the plaques produced on this strain were tiny compared to plaques produced by λ*imm*^{P22}*hy25* on an *ssrA*$^{+}$ strain. In contrast, λ*imm*^{P22}*dis* does not shown any difference in EOP when grown on the *ssrA::cat* strain or the *ssrA::cat-clpP::kan* strain, suggesting that λ*imm*^{P22}*dis* may have additional elements involved in the control of its growth that are not affected by Clp protease.

Measurements of phage burst sizes provided significant ad-

ditional information about the effect of the *clpP::kan* mutation on hybrid-phage growth. As in the EOP experiments, *clpP::kan* suppresses the effect of *ssrA::cat* on the growth of λ*imm*^{P22}*hy25* (Table 2, line 9). This hybrid phage produces a burst in the *clpP::kan-ssrA::cat* strain, K8857, that is 430-fold higher than the burst it produces in the *ssrA::cat* strain, K8619. However, unlike the results observed in the EOP experiments, the burst experiments revealed that *clpP::kan* also suppresses the effect of *ssrA::cat* on λ*imm*^{P22}*dis* growth, although the suppression is considerably less effective than that observed for λ*imm*^{P22}*hy25*. λ*imm*^{P22}*dis* has a burst size in K8857 (*clpP::kan ssrA::cat*) that is 19-fold higher than the burst size it has in K8619 (*ssrA::cat*), while this burst size in K8857 is still 130-fold lower than the burst size produced by λ*imm*^{P22}*dis* in *ssrA*$^{+}$ *E. coli*. These data provide further evidence that λ*imm*^{P22}*dis* may require more than one activity of tmRNA for optimal growth while λ*imm*^{P22}*hy25* requires a single activity of tmRNA, and this activity is unrelated to the degradation of tagged proteins.

Effect of *clpP* mutation in combination with *ssrA*^{O} and *ssrA*^{DD} on growth of λ*imm*^{P22}*dis*. To examine in another way whether *trans*-translation has two functions in the bacterium, we constructed strains carrying the *clpP::kan* allele in the *ssrA*^{O} and *ssrA*^{DD} strain backgrounds. Our results measuring growth of λ*imm*^{P22}*dis* on the *ssrA::cat-clpP::kan* strain suggest that the absence of Clp protease from the bacterium partially suppresses the effect of loss of tmRNA function on λ*imm*^{P22}*dis* growth. Our results measuring both λ*imm*^{P22}*hy25* and λ*imm*^{P22}*dis* growth on *ssrA* mutant strains that produce nondegradable tmRNA tags suggest that *trans*-translation may have dual functions, one being unrelated to degradation of tagged proteins, and that λ*imm*^{P22}*dis* requires both of these functions for optimal growth. We have suggested that a possible explanation for the observed differences in *clpP::kan* suppression between λ*imm*^{P22}*hy25* and λ*imm*^{P22}*dis* is that the absence of only one function of tmRNA must be suppressed for λ*imm*^{P22}*hy25* growth whereas the absence of both functions of tmRNA must be suppressed for optimal λ*imm*^{P22}*dis* growth. Based on these considerations, we predicted that when λ*imm*^{P22}*dis* is grown in a strain supplying one function of tmRNA, absence of Clp protease need only suppress the other function of tmRNA. λ*imm*^{P22}*dis* should thus grow optimally in such a strain.

This hypothesis was tested by introducing the *clpP::kan* allele into the *ssrA*^{O} and *ssrA*^{DD} strain backgrounds and measuring the growth of λ*imm*^{P22}*dis* on these strains. As shown in Table 2, lines 10 and 11, λ*imm*^{P22}*dis* burst sizes on *ssrA*^{O}*-clpP::kan* and *ssrA*^{DD}*-clpP::kan* strains, K9281 and K9282, are significantly higher than the burst sizes on the *ssrA*^{O} and *ssrA*^{DD} strains and only slightly lower than the burst produced by λ*imm*^{P22}*dis* on *ssrA*$^{+}$ *E. coli.* Significantly, the levels of λ*imm*^{P22}*dis* growth measured here are equal to the levels of λ*imm*^{P22}*dis* growth on the *clpP::kan* strain, K8465, suggesting that the absence of Clp protease in the *ssrA*^{O} and *ssrA*^{DD} backgrounds allows nearly optimal λ*imm*^{P22}*dis* growth.

DISCUSSION

The experiments described in this report address the question of whether the *trans*-translation model for tmRNA function can explain the requirement for tmRNA in the growth of certain λ*imm*^{P22} hybrid phages. Our results indicate that the action of *trans*-translation, as originally proposed, is unlikely to explain how this small RNA acts in supporting λ*imm*^{P22} hybrid-phage growth. By postulating that tmRNA acts to remove partially synthesized proteins from the bacterial cell by tagging them for degradation, the model by necessity has four distinguishable components: charging of tmRNA with alanine, release of the stalled translation complex with the nascent peptide from the mRNA template, addition of the tmRNA-encoded tag to the truncated nascent peptide, and degradation of the tagged peptide by cellular proteases.

We begin by discussing the results of our experiments testing whether the first step in the *trans*-translation model, charging of tmRNA with alanine, is required to support the growth of the hybrid phages. The *ssrA* sequence was changed in four ways to alter the nucleotides in the putative acceptor stem of tmRNA that are recognized by the alanyl aminoacyl-tRNA synthetase and are thus required for charging with alanine. One of these mutants, the *ssrA*^{UG} mutant, has conservative changes from the wild type that result in a change from a G · U to a U · G base pair in tmRNA and thus is unlikely to differ significantly in structure from the wild-type molecule. *E. coli* expressing any of these altered tmRNAs as the sole form of tmRNA in the bacterium was unable to support λ*imm*^{P22} growth. The Northern transfer results presented in Table 3 showed that the mutant tmRNAs are found at levels at least as high as the levels of wild-type tmRNA and are therefore sufficient to allow optimal phage growth. These results suggest that it is not the structure of the tmRNA alone that is important for the support of λ*imm*^{P22} growth; the charging of tmRNA with alanine is critical.

Next we discuss the results of our experiments testing whether the *trans*-translation model in its entirety can explain why some λ*imm*^{P22} phages fail to grow in the absence of tmRNA, namely, if tagging and the resulting proteolysis are required for tmRNA to support the growth of these phages. The sequence of tmRNA encoding the peptide tag was changed in two ways to make it ineffective for directing proteolysis. We did this either through the introduction of a nonsense codon, which should terminate tag synthesis upstream of the protease recognition sequence, or by altering the tag sequence in a way previously shown to render it unrecognizable to proteases. Measurement of phage growth by both EOP and burst showed that derivatives of K8619, the *ssrA::cat* strain, having an additional copy of either of the mutant *ssrA* alleles, *ssrA*^{O} or *ssrA*^{DD}, supported growth of λ*imm*^{P22}*hy25* similarly to an isogenic strain with wild-type *ssrA*. The Northern transfer results shown in Table 3 confirm that both of these mutant strains produce stable tmRNAs at or near the level of tmRNA produced in a strain with wild-type *ssrA*. Since these mutant strains express tmRNAs that are unable to add tags appropriate for signaling proteolysis, it is unlikely that tagging peptides for proteolysis plays a significant role in the action of tmRNA in supporting the growth of this phage. However, similar measurements of phage production showed that these derivatives of K8619 provided an intermediate level of support of the growth of the second hybrid phage, λ*imm*^{P22}*dis*. Thus, the action of tmRNA in tagging peptides for proteolysis is likely to be important for optimal growth of λ*imm*^{P22}*dis*. Assuming that the mutant tmRNAs fail to add tags that lead to physiologically significant proteolysis, the fact that there is still partial support of the growth of λ*imm*^{P22}*dis* means that tmRNA must also contribute to the growth of λ*imm*^{P22}*dis* independently of its role in fostering proteolysis.

One possible unifying explanation for the action of the mutant tmRNA in support of the growth of the P22 hybrid phages is that the mutant tag sequences, at some low level, signal proteolysis. Accordingly, this level of proteolysis would be sufficient to support efficient growth of λ*imm*^{P22}*hy25* but only inefficient growth of λ*imm*^{P22}*dis*. Although formally possible, we think that this scenario is unlikely. Experiments from the Sauer laboratory (18) show that peptides tagged with *ssrA*^{DD}

variants are not targets for proteolysis. Moreover, it is difficult to see how the shortened tag presumably added by the *ssrA*^{O} mutant tmRNA could be an appropriate signal, especially since it also ends with an aspartate residue. Finally, it is unlikely that a very low level of proteolysis of proteins carrying the mutant tag sequences could act to foster λ*imm*^{P22} phage growth.

Experiments with a *clpP* mutant provide further support for the conclusion that tagging peptides for proteolysis cannot explain the full role of tmRNA in supporting the growth of the P22 hybrid phages. If degradation of tagged proteins is critical for λ*imm*^{P22} growth, then removing the protease primarily responsible for the degradation should result in a failure in λ*imm*^{P22} growth similar to that seen in an *ssrA::cat* bacterium. We found that growth of the P22 hybrid phages was only slightly decreased in a strain in which the *clpP* gene is disrupted by a kanamycin resistance cassette (*clpP::kan*). Although experiments by Gottesman et al. (10) and Herman et al. (13) provide evidence that Clp is likely to be the protease primarily responsible for degradation of tmRNA-tagged peptides under the conditions of our experiments, it is possible that other proteases can digest tagged proteins in the absence of Clp protease, albeit with a greatly lowered efficiency (10, 13).

If tmRNA has another role in supporting the growth of hybrid phages, what, then, could this role be? Our results with the *ssrA::cat-clpP::kan* double-mutant strain provide some insight into this question. The finding that tmRNA is not required for growth of the hybrid phages in cells that are deficient in Clp protease activity suggests that a protein(s) sensitive to the Clp protease is central to the tmRNA requirement. Accordingly, in the *ssrA::cat* strain, the lack of tmRNA may result in a reduced concentration of this protein, with the level further decreased by Clp-mediated proteolysis, resulting in a concentration of the protein that is insufficient for phage growth. When Clp activity is removed from an *ssrA::cat* cell by introduction of the *clpP::kan* allele, the level of this Clp-sensitive protein increases to a level that supports growth of the hybrid phages. A precedent for such a protein affecting phage growth is the λ-encoded O protein, a substrate for the Clp protease (9, 46). This protein is thought to be limiting for λ growth (22, 25, 47).

Suppression of the effect of *ssrA::cat* by the *clpP::kan* mutation was observed for both hybrid phages tested; however, this suppression was less effective for growth of λ*imm*^{P22}*dis* than for growth of λ*imm*^{P22}*hy25*. This observation suggests that λ*imm*^{P22}*dis* requires an additional function of *trans*-translation for optimal growth and that the *clpP::kan* allele cannot compensate for the loss of both functions of *trans*-translation for λ*imm*^{P22}*dis* growth. The observation that production of λ*imm*^{P22}*dis* in *E. coli* hosts carrying the *ssrA*^{O} and *ssrA*^{DD} alleles is significantly greater than production of this phage in an *ssrA::cat* strain also suggests that degradation of tagged proteins cannot be solely responsible for the failure of λ*imm*^{P22}*dis* to grow in an *ssrA::cat* strain. Experiments measuring λ*imm*^{P22}*dis* bursts in strains carrying both the *clpP::kan* allele and either the *ssrA*^{O} or *ssrA*^{DD} allele show that λ*imm*^{P22}*dis* growth in these strains is similar to λ*imm*^{P22}*dis* growth in a *clpP::kan*-only strain, suggesting that the absence of Clp protease allows nearly optimal λ*imm*^{P22}*dis* growth if one function of tmRNA is provided.

Experiments measuring phage growth in strains lacking Clp protease also suggest two separate functions for Clp in phage growth. The first function is the degradation of tmRNA-tagged proteins, as has been described previously (10, 13, 18). This is shown in our experiments by the approximately twofold decrease in bursts of both hybrid phages in a strain lacking Clp protease. The second function of Clp is one that is detrimental to λ*imm*^{P22} growth in the absence of tmRNA. This is shown by the suppressive effect that *clpP* mutation has on the growth of both hybrids. A likely candidate for this second function, as argued above, is the degradation by Clp of a protein that is limiting for phage growth.

Our previous observation that P22 *c*1 mutations permit both λ*imm*^{P22} hybrids to grow in an *ssrA::cat* strain is consistent with the idea of a limiting phage protein being responsible for the effect of tmRNA on phage growth. We have previously proposed that the binding of C1 protein in the P_R operon reduces expression of downstream functions, including those involved in replication (30, 38). We now suggest that one or more of these functions is a protein that is essential for phage growth, produced in limiting amounts, and sensitive to a protease, most likely Clp. In the presence of C1, while a low level of the limiting protein is expressed, the concentration is sufficient to support phage growth, even with active proteolysis. However, in the absence of tmRNA, lower levels of the protein would be available, and thus it would be reduced to functionally insignificant levels by proteolysis. In the absence of C1, significantly higher levels of the limiting protein would be available and proteolysis would not reduce the protein concentration below the critical level. It should be kept in mind that this proteolysis is unrelated to the proteolysis resulting from tagging by tmRNA.

Model for tmRNA effect on λ*imm*^{P22} growth. The question remains, why would alanine-charged tmRNA be necessary to maintain a critical concentration of a phage product, apart from the role of tmRNA in tagging peptides for proteolysis? Given that tmRNA is known to associate with ribosomes and that alanine charging of tmRNA is required for λ*imm*^{P22} growth and for association of tmRNA with the ribosome, it is conceivable that the lack of functional tmRNA would result in decreased translation of mRNA encoding the limiting protein(s). A model that may explain this effect is shown in Fig. 7. The mRNA encoding the limiting P22 phage product may have a sequence or structure that causes a ribosome to become stalled at some position upstream of the stop codon. The ribosomes following in the polysome would then be blocked from proceeding with translation and stacking of the ribosomes would result, causing a reduction in translation of the limiting protein. In the presence of alanine-charged tmRNA, as shown in Fig. 7A, the stalled ribosome would be removed from the mRNA by *trans*-translation and the following ribosomes would be freed to continue translation. The tagged protein resulting from *trans*-translation may later be digested by proteases, but this degradation step would not significantly affect the expression of the limiting P22 product. In the absence of functional tmRNA, as shown in Fig. 7B, the stalled ribosome would not be removed from the mRNA, translation would be reduced, and levels of the limiting phage product would not rise above the critical concentration. The critical step in *trans*-translation, based on this model, is thus step 2, release of the stalled translational complex from the original mRNA. This model for a mechanism allowing ribosomes to proceed through a translational arrest is not unlike that proposed for RNA polymerase progressing through an arrest site during transcriptional elongation (3).

While evidence exists in support of pieces of this model, many of the details are speculative at this stage. Most notably, it has not been shown whether tmRNA is capable of acting anywhere other than at the 3′ end of a mRNA. It is possible to determine experimentally whether tmRNA has this capability, and such work is currently in progress. Identification of the putative limiting P22 product will also increase our under-

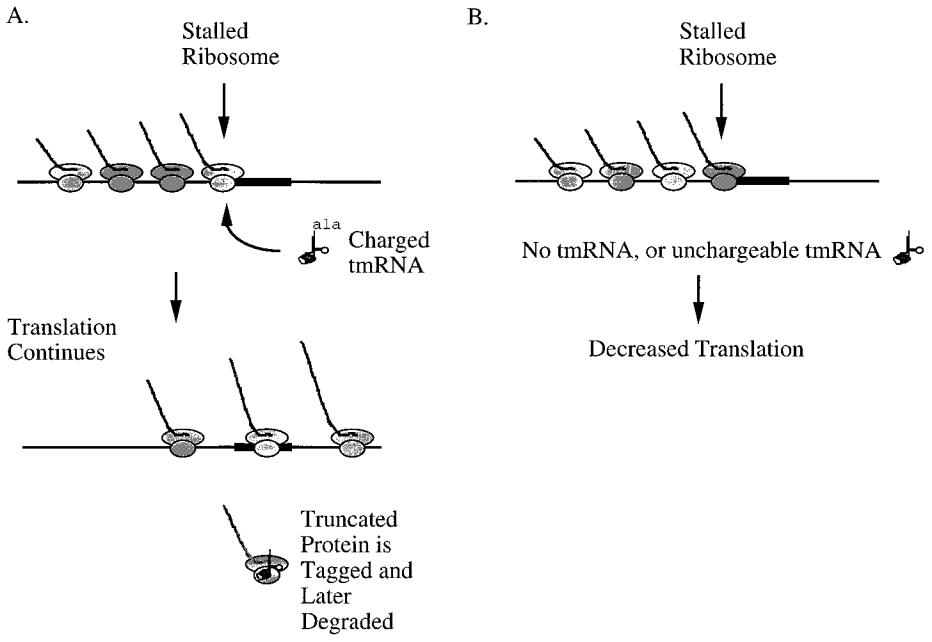

FIG. 7. Model explaining the effect of tmRNA on translation leading to production of a limiting phage product. mRNA is shown as a black line, ribosomes as gray ovals, and nascent proteins as gray lines. A structure or sequence in the mRNA that causes ribosome stalling is indicated by a heavy black line in the mRNA. (A) Translation in the presence of tmRNA action. An alanine-charged tmRNA molecule enters a ribosome that is stalled at a specific structure or sequence in the phage mRNA. The alanine is attached to the nascent peptide, and the ribosome releases the original mRNA. The ribosomes stacked behind the stalled ribosome are then able to continue translation and will be able to traverse the stall site at a frequency that is sufficient for production of the limiting phage protein at a level above the critical concentration. The released ribosome tags its nascent peptide, but this process is not required for phage growth beyond causing release of the stalled ribosome from the mRNA. (B) Translation in the absence of functional tmRNA. When the tmRNA is incapable of being charged with alanine, it cannot enter the stalled ribosome, so a block in translation occurs. This results in insufficient amounts of the limiting phage protein being produced, and the phages are unable to grow.

standing of this system and allow other aspects of this model to be tested. It must be noted that our results suggesting that charged tmRNA is required for phage growth do not rule out the possibility that this charged tmRNA is involved in some process other than *trans*-translation that is required for λ*imm*^{P22} growth.

The model for tmRNA function proposed here, while based on the effects of tmRNA on λ*imm*^{P22} growth, may also apply universally to tmRNA function in bacteria. Work performed in this laboratory on the tmRNA of *Neisseria gonorrhoeae* has shown that it may be essential for survival of the bacterial cell (17a). However, the *ssrA*^{O} allele allows survival when it is present as the only copy of *ssrA* in *N. gonorrhoeae*, so degradation of tagged proteins does not appear to be an important element in this system either. The introduction of an *ssrA* mutation into a *nusA1* strain of *E. coli* yields yet another example of the importance of charging tmRNA with alanine and the relative unimportance of the degradation of tagged proteins. A *nusA1* strain, which affects transcription antitermination of phage λ, supports growth of λ at 32°C, whereas *nusA1-ssrA* double-mutant strains do not support the growth of λ at 32°C (37a). However, the *ssrA* tag mutant alleles, *ssrA*^{O} and *ssrA*^{DD}, are able to support the growth of λ at 32°C in the presence of a *nusA1* mutation while a charging mutant allele, *ssrA*^{UG}, is unable to support λ growth at 32°C in a *nusA1* cell (29a). These findings further support the major thesis advanced here, namely, that degradation of truncated proteins may not be the only role for tmRNA but instead one of two roles, the second being release of stalled ribosomes from their mRNA template, and that this second function may be the more critical of the two.

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