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A Base Substitution in the Amino Acid Acceptor Stem of tRNA^{Lys} Causes Both Misacylation and Altered Decoding

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In 1984, our laboratory reported the characterization of the first misacylated tRNA missense suppressor, a mutant *Escherichia coli* lysine tRNA with a C70 to U base change in the amino acid acceptor stem. We suggested then that the suppressor tRNA, though still acylated to a large extent with lysine, is partially misacylated with alanine. The results reported in this article demonstrate that is the case both in vitro and in vivo. For the in vitro studies, the mutant tRNA species was isolated from the appropriate RPC-5 column fractions and shown to be acylatable with both lysine and alanine. For the in vivo demonstration, use was made of a temperature-sensitive *alaS* mutation, which results in decreasing acylation with Ala as the temperature is increased, resulting ultimately in lethality at 42°C. The *alaS*^{ts} mutation was also used to demonstrate that the ability of the same missense suppressor, *lysT*(U70), to suppress a *trpA* frameshift mutation is not affected by the Ala-acylation deficiency. We conclude that the misacylation and altered decoding are two independent effects of the C70 to U mutation in tRNA^{Lys}. The influence of an alteration in the acceptor stem, which is in contact with the large (50S) ribosomal subunit, on decoding, which involves contact between the anticodon region of tRNA and the small (30S) ribosomal subunit, may occur intramolecularly, through the tRNA molecule. Alternatively, the U70 effect may be accomplished intermolecularly; for example, it may alter the interaction of tRNA with ribosomal RNA in the 50S subunit, which may then influence further interactions between the two subunits and between the 30S subunit and the anticodon region of the tRNA. Preliminary evidence suggesting some form of the latter explanation is presented. The influence of a single nucleotide on both tRNA identity and decoding may be related to the coevolution of tRNAs, aminoacyl-tRNA synthetases, and ribosomes.

Acceptor stem mutation   G3·U70   tRNA identity   Frameshifting   Suppression

TRANSLATIONAL suppression is a most effective way to examine the structure, function, and interactions of any translational macromolecule, as long as and to the extent that that molecule is involved in the specificity or accuracy of translation. Very useful mutations for the study of translational mechanisms have been found in tRNAs, elongation factors, peptide chain termination factors, ribosomal proteins, and ribosomal RNAs [for review, see (34) and references therein]. Until the early 1980s, however, the only well-characterized translational suppressors of missense mutations in *Escherichia coli* were mutant tRNAs whose codon recognition properties had been changed either by nucleotide substitution in the anticodon or by nucleotide insertion in the anticodon loop outside of the anticodon [reviewed in (33)]. One particular mechanism proposed in the original hypothesis to explain missense suppression had not been observed, namely alteration of a tRNA such that it is misacylated but retains its original decoding specificity (3,68). In 1978, however, Hadley and Murgola reported the isolation of missense suppressors that were very strong can-

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didates for this class (14). They were isolated as suppressors of *trpA*(AAG211), a missense mutation consisting of the Lys codon AAG in place of the Gly codon GGA at the mRNA codon position corresponding to amino acid 211 of the protein product of the *trpA* gene. Based on their roughly determined map location, distant from any glycine tRNA genes, and on their specificity for the Lys codon AAG, it was suggested that the suppressors might be misacylated lysine tRNAs.

In a later study we demonstrated that the AAG suppressors also read the other Lys codon, AAA, and presented strong evidence that they are mutant Lys tRNAs, misacylated with either Gly or Ala (36). In 1984, our laboratory reported the purification and nucleotide sequence analysis of the mutant Lys tRNAs from two of the $AA^{A}/_{G}$ suppressor strains (48). The results were the same in both cases and demonstrated that missense suppression can be caused by nucleotide substitution in the amino acid acceptor stem of Lys tRNA (Fig. 1a). The mutational change was the substitution of U for C at residue 70, resulting in the conversion from G-C to G·U of the third base pair from the acceptor end (Fig. 1a, b). In that report, we compared the acceptor stem of the mutant Lys tRNA with that of Gly and Ala tRNAs. On two points in particular the mutant tRNA was strikingly similar to both Ala tRNAs (Fig. 1c), $tRNA^{Ala}_{VGC}$ (25,69) and $tRNA^{Ala}_{GGC}$ (31), but different from $tRNA^{Gly}$. First, nucleotide 73, the fourth from the 3′ end, was A in the Ala tRNAs, as is true of Lys tRNA, but U in all three Gly isoacceptors. This nucleotide was designated the "discriminator" nucleotide (8) and has now been verified

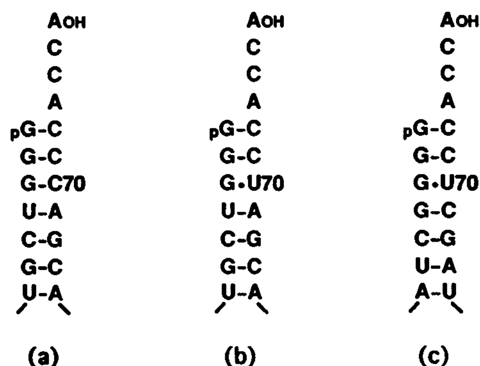

FIG. 1. Diagram of the amino acid acceptor stems of: (a) wild-type *E. coli* $tRNA^{Lys}$ (6,48); (b) mutant $tRNA^{Lys}$, containing the C70U mutation that allows it to be misacylated with Ala [(48); this study] and to cause suppression of the "+1" frameshift mutation *trpA8* (63); and (c) both $tRNA^{Ala}{}_{VGC}$ (25,69) and $tRNA^{Ala}{}_{GGC}$ (31). Hydrogen bonding in standard base pairs (-); hydrogen bonding in a nonstandard base pair (·).

as having an important role in determining tRNA identity [reviewed in (55)]. Second, the U-for-C substitution in Lys tRNA created a G3·U70 base pair. That base pair is present in the Ala tRNAs but not in the Gly tRNAs, and in fact is characteristic of all cytoplasmic Ala tRNAs (13). An earlier study by Schulman and Chambers (56) had suggested that the acceptor stem of Ala tRNA might indeed be important for acylation with Ala. Later studies with Ala tRNA exposed the G3·U70 base pair as the major determinant of Ala identity (17,30), a result consistent with and supportive of our analyses of the Lys tRNA missense suppressors. That left to us only to directly demonstrate the misacylation with Ala.

In 1989, we reported that the *lysT*(U70) missense suppressor, in addition to other missense suppressors, could also suppress two +1 frameshift mutations in *trpA* (63). The *lysT*(U70) suppressor was particularly noteworthy because the single alteration in the Lys tRNA was the C-to-U base change in the amino acid acceptor stem, far from the anticodon end. However, the virtual certainty that it was a misacylated tRNA raised the question of whether the frameshifting was indeed the property of the mutant Lys tRNA but not of the wild-type species. One alternative to the former is that the wild-type tRNA can perform +1 frameshifts but the suppression seen with the *trpA* mutants requires the insertion of Ala rather than Lys into the polypeptide at the site of frameshifting. Another alternative is that neither the wild-type nor the mutant tRNA actually *does* the frameshifting, but rather that the misacylated mutant tRNA causes some physiological "unrest" that leads to frameshift suppression. An example of the latter would be the production of ribosomes that make more translocation mistakes due to the substitution of Ala for Lys in critical ribosomal proteins. Both alternative explanations depend on misacylation of the mutant Lys tRNA with Ala.

The results reported in this article demonstrate that a) the mutant *lysT*(U70) tRNA, though still acylatable substantially with Lys, can be misacylated in vitro with Ala; b) missense suppression in vivo requires misacylation of the mutant tRNA with Ala; and c) frameshift suppression by *lysT*(U70) tRNA does not depend upon misacylation with Ala.

MATERIALS AND METHODS

Bacterial Strains and Procedures

The main *E. coli* K12 strains used in this study are described in Table 1. The nomenclature for

TABLE 1
BACTERIAL STRAINS USED IN THIS STUDY

Strain	Genotype
FTP5129	*lysT*(U70) *trpA*(AAG211) *trpR glyV55*
FTP5133*	*lysT*(U70) *trpA8 trpR glyV55*
FTP5157†	*glyT*(SuAA^A/$_G$) *trpA*(AAG211) *trpR glyV55*
FTP5230*	*alaS5 lysT*(U70) *trpA8 trpR glyV55*
FTP5237	*alaS5 lysT*(U70) *trpA*(AAG211) *trpR glyV55*
FTP5240†	*alaS5 glyT*(SuAA^A/$_G$) *trpA*(AAG211) *trpR glyV55*
FTP5258‡	*alaS5 lysT*(U70) *trpA*(GUA211) *glyV55*
FTP5261	*alaS5 lysT*(U70) *trpA*(AAG211) *glyV55*
FTP5265*	*alaS5 lysT*(U70) *trpA8 glyV55*
FTP5267†	*alaS5 glyT*(SuAAA/G) *trpA*(AAG211) *glyV55*
KL380§	*alaS5 recA1 leuB6 argA21 metE70 ara-14 lacI3 lacZ118 xyl-5 mtl-1 rpsL104 supD*

**trpA8*, a +1 frameshift mutant, is described in Tucker et al. (63).
†*glyT*(SuAA^A/$_G$) is described in Murgola et al. (37).
‡*trpA*(GUA211) is a non-wild-type TrpA$^+$, obtained as a revertant of the missense mutant *trpA*(GAA211).
§From P. Schimmel. *alaS5* [isolated by Theall et al. (61)] = "*alaS*^{ts}." It is a Gly677 to Asp missense mutant (19).

trpA mutations and suppressor genes has been described (33), as have the growth media and procedures for examining relative growth in liquid and on solid media (4,32,35,37,40,63).

tRNA Isolation and In Vitro Aminoacylation

Procedures for growing cells, preparing crude tRNA, isolation of tRNAs by reversed phase column chromatography (RPC-5) and polyacrylamide gel separation, and RNA sequence analyses have been previously described (33,39,48). Two new mutant derivatives of *lysT*(U70) tRNA were isolated for sequence analysis according to modifications of those procedures. Bulk tRNA preparations were chromatographed on an RPC-5 column. The tRNAs from pooled fractions were recovered and separated on a partially denaturing (4 M urea) 20% (w/v) polyacrylamide gel. Further purification on a 12% polyacrylamide denaturing gel (7.5 M urea) was sometimes necessary. One mutant, the *lysT*(SuUAA/G), was identified using a Northern-type analysis in which tRNA species separated on the 20% gel were electroblotted in Tris/borate/EDTA, pH 8.3, onto Immobilon N membrane from Millipore Corp. using a Hoefer TE50 Transphor transfer unit. Subsequent hybridization to an 18-residue oligodeoxyribonucleotide complementary to nucleotides 32–49 of tRNA^{Lys} indicated the location of the mutant species in the gel. The oligonucleotide was purified on a 19% polyacrylamide gel, visualized by UV shadowing, and eluted from the gel using procedures of Sambrook et al. (52). It was phosphorylated with [γ-^{32}P]ATP using T4 polynucleotide kinase. Postlabeling purification was done using NACS PREPAC nucleic acid purification cartridges from Bethesda Research Laboratories according to the manufacturer's recommended procedure.

For RPC-5 column analysis of in vitro aminoacylation, the source of lysyl-tRNA synthetase (LysRS) was an S-100 preparation from wild-type *E. coli*. Alanyl-tRNA synthetase (AlaRS) was prepared as a partially purified extract from cells containing the *alaS* gene cloned into a high-copy plasmid (from P. Schimmel). Aminoacylation reaction conditions were as follows: for lysine: piperazine-*N,N'*-bis[2-ethanesulfonic acid], pH 7.0, 50 mM; $MgCl_2$, 15 mM; KCl, 10 mM; β-mercaptoethanol, 12 mM; bovine serum albumin, 0.2 mg/ml; and ATP, 4 mM; and for alanine: sodium phosphate monobasic, pH 7.5, 40 mM; $MgCl_2$, 10 mM; KCl, 20 mM; dithiothreitol, 5 mM; BSA, 0.1 mg/ml; and ATP, 4 mM. ^{14}C- or ^{3}H-labeled lysine and alanine were used at appropriate specific activities.

Tryptophan Synthetase Assays

To quantitate missense and frameshift suppression, we assayed crude extracts for the activities of the two subunits, α and β, of tryptophan synthetase, the terminal enzyme in the biosynthetic pathway to Trp. The β subunit is encoded by *trpB* and the α subunit by *trpA*, the gene in which the suppressible mutations are located. In this study we used a radioactive version of the classical assays (59). In each set of assays, we normalized the specific activity of α to that of β in the same extract. That ratio was then expressed as a percentage of the same ratio for the *trpA*$^+$ control strain, whose extract was prepared and assayed at the same times as the test extracts.

Effect of 16S rRNA Mutations on Missense Suppression by lysT*(U70) tRNA*

To examine whether the allelic state of 16S rRNA can influence the missense suppressor activity of the *lysT*(U70) mutation, we compared suppressor tRNA strains containing the plasmid pNO2680 (11), which carries the wild-type version of *rrnB*, one of the operons for rRNA (16S, 23S, and 5S), with suppressor strains containing plasmids with two different rRNA mutations. The mutations were in the 16S gene, namely, C1054A and C1054Δ. The first mutant suppresses nonsense codons and is lethal when highly expressed at high temperatures; the second does not suppress nonsense mutations and is lethal when highly expressed at any temperature [(38); F. T. Pagel, S. Q. Zhao, K. A. Hijazi, and E. J. Murgola, submitted]. The strains were examined for growth on glucose minimal medium (i.e., for suppression of an AAG mutation in *trpA*).

RESULTS

In Vitro Aminoacylation

In general, crude preparations of total tRNA from *lysT*$^+$ or *lysT*(U70) strains were fractionated through a salt gradient of 0.4 to 0.67 M NaCl on an RPC-5 column. In one type of experiment, one aliquot of total tRNA from a *lysT*(U70) strain was acylated with [^{3}H]Lys, and another with [^{14}C]Ala, and both were cochromatographed on an RPC-5 column. In the fractions eluted ahead of the normal Lys and Ala peaks, we observed a single small Ala peak coincident with a single Lys peak. Such a Lys peak did not appear in radiolabeled Lys-tRNA profiles of total *lysT*$^+$ tRNA, and its location was precisely that from which the *lysT* suppressor tRNA was originally isolated [(48); B. H. Mims, personal communication].

To examine further the Lys- and/or Ala-acylatable species in that peak, we acylated total tRNA from a *lysT*$^+$ strain and that from a *lysT*(U70) strain with [^{3}H]Ala and fractionated both radiolabeled preparations separately. In the fractions of interest, ahead of the normal Ala profile, the *lysT*(U70) total tRNA again displayed a very small Ala peak. However, the corresponding fractions of the *lysT*$^+$ tRNA preparation did not. The tRNA in that small [^{3}H]Ala-labeled peak of the former preparation and in the corresponding fractions of the latter was recovered (separately from the two preparations) and subjected to deacylation (Tris, 0.2 M; pH 7.5). One sample was then acylated with [^{3}H]Ala and the other with [^{14}C]Ala and the two were cochromatographed on an RPC-5 column. The result was the appearance of a small peak of radioactivity with the sample from the *lysT*(U70) strain but none with the sample from the *lysT*$^+$ strain (Fig. 2A). We then examined further the deacylated, individually fractionated total tRNA from the *lysT*(U70) strain by acylating one aliquot with [^{3}H]Ala and another with [^{14}C]Lys and cochromatographing the two. This resulted again in one small Ala peak entirely coincident with one larger Lys peak (Fig. 2B).

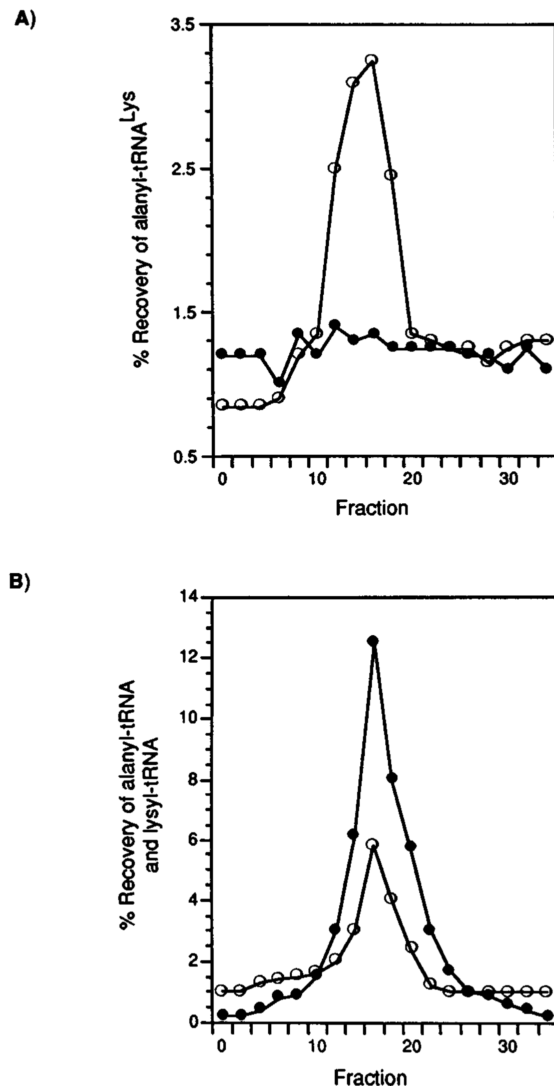

FIG. 2. (A) Cochromatography, following aminoacylation with radiolabeled Ala, of tRNA obtained from the early eluting Lys peak from the suppressor [*lysT*(U70)] strain (○, [^{3}H]Ala) and tRNA from pooled fractions of the corresponding region of the *lysT*$^+$ profile (●, [^{14}C]Ala). (B) Cochromatography, on RPC-5, of aliquots of partially purified *lysT*(U70) tRNA acylated with (○) [^{14}C]Ala or (●) [^{3}H]Lys.

In Vivo Misacylation

If the missense suppressor activity of *lysT*(U70) depends upon the capacity for misacylation with Ala, as demonstrated in the preceding section, then limitation of Ala acylation should limit missense suppression. To demonstrate this in vivo, we made use of a mutant allele of *alaS* that encodes a temperature-sensitive AlaRS (61). As the incubation temperature for a strain containing this allele (*alaS5*; "*alaS*^{ts}") is raised, the AlaRS activity in the cell decreases; at 42°C, the mutation is lethal. We reasoned that if *lysT*(U70) tRNA is misacylated with Ala in vivo and that misacylation is required for the missense suppression, then at some temperature below 42°C we might observe cessation of the missense suppression while cell viability is still maintained.

Table 2 presents the results of such an experiment. A strain was constructed (FTP5261) with the suppressor, *lysT*(U70), the suppressible missense mutation, *trpA*(AAG211), and the *alaS*^{ts} allele. Constructed also were two isogenic control strains: FTP5258 has *trpA*$^{+}$ in place of the missense mutation, and FTP5267 has in place of the *lysT* missense suppressor a tRNA^{Gly} missense suppressor that reads the same codons, AAA and AAG. That *glyT*(SuAA^{A}/$_{G}$), however, should not depend upon *alaS* for its suppressor activity. The growth of these three strains on glucose minimal medium (a measure of suppression) was examined at four temperatures. As expected, because of the high-temperature conditional lethality of *alaS*^{ts}, at the highest temperature, 42°C, none survived (even on medium containing Trp). At the other three temperatures, 31°, 35°, and 37°C, the two control strains behaved as expected, namely, the higher the temperature (in that range), the better the growth. FTP5261, however, the test strain for *lysT*(U70) missense suppression, exhibited less growth (i.e., suppression) the higher the temperature, and no growth, finally, at 37°C (even after 6 days), even though at that temperature it grew on medium containing Trp.

To better quantitate this Ala-dependent missense suppression, we constructed pairs of strains for tryptophan synthetase assays [as in (59)]. These strains are displayed in Table 3. One member of each pair was *alaS*$^{+}$, the other *alaS*^{ts}. A similar pair of strains was prepared with *trpA*$^{+}$, and their assay values were used in the calculation of percent suppression. The extracts used in the assays were made from cells grown in the presence of Trp at 37°C, a temperature suggested as appropriate by the results in Table 2. Whereas the ts/tr ratio of the *glyT*(SuAA^{A}/$_{G}$) control strains was not below 1, that of the *lysT*(U70) strains was essentially zero (Table 3).

Frameshift Suppression

To determine whether misacylation with Ala is necessary for the *lysT*(U70)-induced suppression of the +1 frameshift mutant *trpA8*, we examined the appropriate *trpA8* strains for growth in the absence of Trp (an indication of suppression) at different temperatures and for efficiency of suppression at 37°C in the tryptophan synthetase assays. As Table 2 indicates, the *alaS*^{ts} allele did not alter the growth of *lysT*(U70)-suppressed *trpA8* at sublethal temperatures; indeed, the strain grew better the higher the temperature. Table 3 indicates that the suppressed *trpA8* strain behaved just like the *glyT*(SuAAG) control missense suppressor strain; that is, the suppression was not af-

TABLE 2
RELATIVE GROWTH* AT DIFFERENT TEMPERATURES OF SUPPRESSOR STRAINS CONTAINING A TEMPERATURE-SENSITIVE ALANYL-tRNA SYNTHETASE

Strain†	Relevant Genotype†			Temperature			
				31°	35°	37°	42°
FTP5258	*alaS*^{ts}	*lysT*(U70)	*trpA*$^{+}$	2‡	3	4	0
FTP5267	*alaS*^{ts}	*glyT*(SuAAG)	*trpA*(AAG211)	1	2	2.5	0
FTP5261	*alaS*^{ts}	*lysT*(U70)	*trpA*(AAG211)	1.5	0.2	0	0
FTP5265	*alaS*^{ts}	*lysT*(U70)	*trpA8*	0.5	1	1.5	0

*Relative growth was determined both by comparison on glucose minimal agar medium (after 3 days) and by observing growth rates in liquid glucose minimal medium. The ability to grow in the absence of Trp is an indication of the suppression of the *trpA* missense and frameshift mutations relative to *trpA*$^{+}$, which requires no suppression to grow in the absence of Trp.

†See Table 1 for additional information.

‡The higher the number, the better the growth. The *trpA*$^{+}$ strain was recorded after 1 day on plates. None of the four strains grew at 42°, even in the presence of Trp. At 37°, FTP5261 grew as well as the others on medium containing Trp.

TABLE 3
EFFECT OF A TEMPERATURE-SENSITIVE ALANYL-tRNA SYNTHETASE ON MISSENSE AND FRAMESHIFT SUPPRESSION BY *lysT*(U70)

Strain*	Relevant Genotype*			Percent Suppression† (37°)	ts/tr‡
FTP5240	*alaS*^{ts}	*glyT*(SuAA$^{A}/_{G}$)	*trpA*(AAG211)	0.52	1.5
FTP5157	*alaS*$^{+}$	*glyT*(SuAA$^{A}/_{G}$)	*trpA*(AAG211)	0.33	
FTP5237	*alaS*^{ts}	*lysT*(SuAA$^{A}/_{G}$)	*trpA*(AAG211)	0.00§	0
FTP5129	*alaS*$^{+}$	*lysT*(SuAA$^{A}/_{G}$)	*trpA*(AAG211)	0.55	
FTP5230	*alaS*^{ts}	*lysT*(SuAA$^{A}/_{G}$)	*trpA8*¶	0.2	1.5
FTP5133	*alaS*$^{+}$	*lysT*(SuAA$^{A}/_{G}$)	*trpA8*	0.13	

*See Table 1 for additional information.

†Determined by radioactive tryptophan synthetase (TS) assays. The specific activity of the TS α subunit was normalized to that of the β subunit and then that ratio expressed as a percentage of the similar ratio for an isogenic *trpA*$^{+}$ strain (see Materials and Methods section for additional details).

‡Ratio of the percent suppression of the ts member to that of the tr (temperature resistant, that is, *alaS*$^{+}$) member of each pair.

§At the limit of sensitivity of the assay (ca. 0.03%).

¶A +1 frameshift mutation; see Table 1.

fected by the presence of *alaS*^{ts} at the temperature (37°C) at which missense suppression is abolished.

A tRNA Identity Determinant and Decoding

The results presented in the preceding section demonstrate that the misacylation caused by U70 is not responsible for the observed frameshift suppression of *trpA8*. Therefore, the U70 is directly responsible for the altered decoding (frameshifting). This causal connection could be intramolecular, through the tRNA molecule, or intermolecular, by way of a U70-induced interaction with another translational macromolecule, which in turn would affect decoding, as, for example, elongation factor G or one of the ribosomal RNAs. To investigate further the relationship between U70 and decoding by tRNA^{Lys}, we asked four questions:

1. Can tRNA^{Lys} retain U70 and be converted to new decoding specificities?
2. If so, does U70 influence the accuracy of those new specificities?
3. Does U70 cause the new mutants to frameshift (i.e., suppress frameshift mutations)?
4. Can we provide any indication that the decoding effect of the mutant tRNA occurs by way of interaction with the ribosome?

For the in vivo conversion of *lysT*(U70) tRNA, we used suppression of mutant codons at *trpA* position 234 as our selective phenotype. We presumed that that would insure the retention of U70 because only Gly and Ala are functional at amino acid position 234 of the *trpA* protein (33). Selections for both spontaneously occurring and ethylmethanesulfonate-induced mutations produced new mutant alleles of *lysT*(U70), ones that cause suppression of the codons AGA and AGG, UGA and UGG, GAA and GAG, or UAA and UAG. Nucleotide sequence analysis was performed directly on the mutant tRNAs so we could evaluate the nature and status of modified nucleosides. For the isolation of two of the new mutant tRNAs (the AG$^{A}/_{G}$ and GA$^{A}/_{G}$ readers) for sequence analysis, gel purification proved sufficient. The UA$^{A}/_{G}$ suppressor required use of Northern blots involving a ^{32}P-labeled oligodeoxyribonucleotide complementary to the anticodon region of tRNA^{Lys} (see the Materials and Methods section). These three new mutants arose from the predictable base changes in the anticodon and retained the original U70 mutation. The UG$^{A}/_{G}$ suppressor was not sequenced. Table 4 summarizes these mutants. To examine the codon specificities of the new suppressors, we tested them for their ability to suppress mutant codons related to their cognate codons by a single base difference. Each suppressor appeared to be entirely specific for its cognate codons. Furthermore, none suppressed the frameshift mutant *trpA8*.

Finally, in an attempt to provide an indication that the U70 tRNA^{Lys} may alter decoding by interacting differently with the ribosome, we asked whether mutant alleles of rRNA have perhaps different effects on missense suppression by the mutant tRNA. To this end, we introduced into a strain containing an AAG mutation in *trpA* and *lysT*(U70) each of three plasmids, one containing wild-type rRNA genes and two containing mutations in the 16S rRNA gene (see the Materials and

TABLE 4
MUTANT LYSINE tRNAs

Suppressor	Codons Read	Mutant Nucleotides 34*	35	36	37 . . . 70	Origin/Remarks
lysT+ (wild-type)	AAA, AAG	U_8†	U	U	A_7† . . . C	
lysT(U70; SuAA^A/$_G$)	AAA, AAG	U_8	U	U	A_7 . . . . ***U***	Single base change from *lysT*+; partially misacylated with Ala
lysT(U70; SuAG^A/$_G$)	AGA, AGG	U_8	***C***	U	A_7 . . . . ***U***	One step from *lysT*(U70; SuAA^A/$_G$)
lysT(U70; SuUG^A/$_G$)	UGA, UGG	Not determined				From *lysT*(U70; SuAG^A/$_G$)
lysT(U70; SuGA^A/$_G$)	GAA, GAG	U_8	U	***C***	(A_2)‡ . . . ***U***	One step from *lysT*(U70; SuAA^A/$_G$)
lysT(U70; SuUA^A/$_G$)	UAA, UAG	U_8	U	***A***	A_5 . . . ***U***	One step from *lysT*(U70)

*Nucleotides 34, 35, and 36 constitute the anticodon; nucleotide 70 is in the amino acid acceptor stem. Mutant nucleotides are in bold italics.

†U_8, 5-methylaminomethyl-2-thiouridine (mam^{5}s^{2}U); A_7, *N*-[(9-β-D-ribofuranosylpurin-6-yl)carbamoyl]threonine (t^{6}A); A_2, 2-methyladenosine (m^{2}A); A_5, 2-methylthio-*N*^{6}-isopentenyladenosine (ms^{2}i^{6}A).

‡Parentheses indicate that the nucleoside is predominantly unmodified.

Methods section). The results, shown in Table 5, indicate that the two rRNA mutations had different effects on missense suppression by the U70 tRNA^{Lys}.

DISCUSSION

The results presented here demonstrate, both in vitro and in vivo, that the missense suppressor *lysT*(U70) tRNA is misacylated with Ala while still being acylatable with Lys, and that its missense suppressor activity requires acylation with Ala. In our results with *trp* enzyme assays (Table 3), an interesting aside arises from the comparison of the two members of each pair of strains, one containing the temperature-sensitive AlaRS and the other the wild-type synthetase. The primary point is that the ts/tr ratio of the *lysT* missense suppressor pair gave a ratio of 0. The control suppressor strains, however (i.e., those with the *glyT* missense suppressor), gave a ratio that was not 1 or perhaps a little less than 1, but rather a ratio *higher* than 1 (it can also be seen that, in this respect, the frameshift suppression strains behaved like the control missense suppressor strains). It is not clear whether this difference from the expected is related to a general cellular effect or to one specific to translation of *trpA* mRNA. However it clearly indicates a positive effect on protein synthesis. The effect could be due to a specific role of one of the Ala tRNAs, perhaps by way of an unacylated version; second, it could be caused by alleviation of misacylation of some non-Ala tRNA, which then increases the cognate acylation of that tRNA; or, finally, it may imply a role for 10Sa RNA (22) in normal polypeptide elongation in addition to turnover of truncated proteins (21,62).

Our results also demonstrate that the ability of *lysT*(U70) tRNA to suppress the frameshift mutant *trpA8* does not require acylation with Ala. As suggested in the Introduction, one could imagine that while the wild-type tRNA^{Lys} might be able to perform the necessary frameshift, insertion of Lys might not yield a functional TrpA protein. That possibility may seem likely when one considers the unusual structure of the anticodon region of this

TABLE 5
EFFECT OF MUTANT *rrsB* ALLELES ON SUPPRESSION BY MUTANT tRNAs

Suppressor tRNAs	Ribosomal RNA Alleles *rrsB*+	*rrsB*(A1054)	*rrsB*(ΔC1054)
lysT(SuAA^A/$_G$)	[-]	↓	–
glyU(SuGA^A/$_G$)	[-]	–	–

↓, decrease of suppressor tRNA activity in the presence of the rRNA mutation; –, no effect of the rRNA mutation on the suppressor tRNA.

tRNA and its involvement in programmed frameshifting (66). Nevertheless, there is a way of detecting full-length TrpA independent of its catalytic activity (which would be an indication that wild-type $tRNA^{Lys}$ can cause the appropriate compensatory frameshift event), namely growth on glucose minimal medium supplemented with a very low concentration of indole and a high concentration of 5-methyl-DL-tryptophan [Ind-5MT; see (34) for description]. The *lysT*$^+$ strain containing *trpA8*, however, is unable to grow on Ind-5MT. That result is not shown here, but it appears in Table II of Tucker et al. (63). That same study characterized a spontaneous revertant of the "+1" frameshift mutant *trpA8*, one in which a frame-compensating deletion of one nucleotide occurred 34 nucleotides downstream of the original insertion mutation. That mutant, *trpA8*(PR1), was able to grow on Ind-5MT even though it created a roughly 10-amino acid missense sequence. We conclude, therefore, that wild-type $tRNA^{Lys}$ does not possess precisely the frameshifting ability displayed by the U70 mutant.

Because we assayed only for misacylation with Ala, one may wonder whether continued frameshift suppression in the presence of *alaS*^{ts} at high temperature (see Table 2, line 4, and Table 3, line 5) is the result of misacylation of the U70 tRNA with some other amino acid. Although this is not yet entirely ruled out, we consider its likelihood to be minimal in the light of the following considerations. First, transfer of the G3·U70 base pair to other tRNAs resulted in misacylation with only Ala (17,30). Second, attempts to aminoacylate the Ala minihelix with other synthetases and their cognate amino acids failed (9,26,53). Third, the amber-suppressor derivative of $tRNA^{Lys}$ containing the G3·U70 base pair was acylated with no amino acid other than Ala (29). That result, however, must be tempered by recogniton that the assay used is not sensitive enough to detect some misacylation, because amino acid sequence analysis of the protein dihydrofolate reductase (DHFR) has a rather high noise level (ca. 5%). Supporting that caveat is our unpublished result with the amber-suppressor derivative of $tRNA^{Cys}$. That tRNA was reported to insert only Cys at position 10 in DHFR (17,43). Nevertheless, we were able to observe suppression of the nonsense mutation *trpA*(UAG234), even though Cys is not functional at amino acid position 234 in the TrpA protein (33). In addition, only Gly and Ala are functional at 234, and a replacement efficiency of as little as 1% is sufficient to generate enough TrpA protein for the cells to grow on minimal medium in the absence of Trp. Therefore, as an aside, we conclude that the $tRNA^{Cys}$ amber suppressor is misacylated to a small extent with either Gly or Ala. A final reason for thinking that the *lysT*(U70) tRNA is not aminoacylated with any other amino acid requires recalling that, at position 211 of the TrpA protein, seven amino acids other than Ala and the wild-type Gly (Cys, Ser, Thr, Ile, Leu, Val, and Asn) render the protein catalytically active (33). Therefore, if the U70 tRNA could also be misacylated with one of those amino acids, the missense suppression reported in Table 2 (line 3) and Table 3 (line 3) would not have been affected by high temperature in the presence of the ts allele of *alaS*.

These considerations, taken with the evidence cited at the beginning of this section that wild-type $tRNA^{Lys}$ does not cause an appropriate compensatory frameshift, allow the conclusion that the single base change in the amino acid acceptor stem of $tRNA^{Lys}$ causes both misacylation and altered decoding, and that it causes the latter directly, independently of the misacylation with Ala. In hindsight, *lysT*(U70) provided the first mutational evidence for the importance of G3·U70 for $tRNA^{Ala}$ identity. From the frameshifting analysis the major conclusion is that the C70 to U change gives the mutant tRNA its frameshifting ability. The converse type of mutant tRNA, reported 22 years ago (67), arose from a base substitution in the anticodon of $tRNA^{Trp}$ that converted it from a UGG reader to a UAG reader and simultaneously caused the tRNA to be misacylatable with Gln.

In 1979, Kurland proposed that misreading of a codon would increase the probability of errors of translocation (23). [This predicted occurrence of frameshift events as a consequence of codon misreading was later referred to as error coupling (24).] The "misreading" described by Kurland (23) was of two types. In the first type, mismatches (lack of complementarity between codon and anticodon) result in misreading in the strict sense. In the second type, however, the codon being read and the anticodon of the translating tRNA match, when considered simply as antiparallel, hydrogen-bonded trinucleotides, but the tRNA molecule as a whole displays a conformational perturbation or structural difference of some sort that in turn affects the ribosome-dependent interaction with the codon. We have referred to tRNAs of the latter type as "conformational misfits" and those of the former type as "inept match makers" (63). For several tRNA suppressors of *trpA8* in the Tucker et al. study (63), there were no cognate codons in either the 0 or the +1 reading frame. Therefore, it is likely that those suppressors work by misreading

in the strict sense. The site of frameshifting by *lysT*(U70) in *trpA8* mRNA has not been determined, but it seems likely that it is at one of two lysine codons present in the zero frame in the suppression window (63). If that proves to be the case, *lysT*(U70) would represent an example of a conformational misfit, a tRNA that is cognate to the in-frame codon at the frameshift site but whose conformation is in some way different from the wild-type (caused in this case by U70 or the G3·U70 base pair), leading to an increased likelihood of frameshifting.

It was a little surprising that none of the new suppressor derivatives of *lysT*(U70) suppressed the *trpA8* frameshift mutation. That mutation is suppressed by $tRNA^{Gly}$ suppressors of AGA, UGG, and GAA (63) and therefore contains sites within its suppression window at which the new *lysT* mutants could theoretically shift frame. One possibility is that the mutation in the anticodon of $tRNA^{Lys}$ may, in conjunction with the C70U change, render the tRNA unable to shift at those sites. Such a differential interaction of anticodon changes with changes in the acceptor stem have been noted for glycine tRNAs with respect to acylation specificity (27); a similar case may obtain for decoding with certain tRNAs. Another consideration is that the new suppressor derivatives differ at the 3′-adjacent nucleotide 37. It has been shown that, in $tRNA^{Lys}$, an interaction between the hypermodified nucleosides mam^5s^2U34 and t^6A37 imparts a structure different from the unmodified anticodon and different from a similar anticodon region with the same modified nucleotide 34 but with ms^2i^6A at 37 [reviewed in (1)].

The *lysT*(U70) suppressor tRNA is particularly significant in that the decoding effect is produced by a base substitution so far from the anticodon. O'Mahony et al. (46) reported frameshift suppression by a mutant glycine tRNA altered in the acceptor stem, at the 5′ nucleotide (G1 to A1). That mutant was not characterized at the tRNA level but that nucleotide has been implicated in tRNA identity for the Gly family (27). [For other very interesting frameshift suppressors, see (2).] Interesting acceptor stem mutations have been described in tRNAs that read rare codons for Leu or Arg [see (7), and references therein]. But in those cases, the mutations appear to impair the general translational ability of the tRNAs rather than to change their decoding specificities. For many years, the well-studied "Hirsh suppressor" was considered unique, a tryptophan tRNA UGA suppressor that arose from a base substitution, not in the anticodon but in the stem of the D arm (15). That G-to-A change at residue 24 was later shown by Vacher and Buckingham (64) to cause a conformational change in the tRNA, which could mediate transmission of an effect from nucleotide 24 to the anticodon region. Smith and Yarus (58), however, obtained results supporting the hypothesis that the altered decoding by the mutant $tRNA^{Trp}$ is caused by an altered interaction with the ribosome. In this study, we have provided preliminary evidence that *lysT*(U70) tRNA responds to AAG differently depending on the nature of a cloned 16S rRNA allele present in the same cell. This result is particularly interesting because U70 is in the amino acid acceptor stem, very close to the -CCA_{OH} end of the tRNA, which is in contact with 23S rRNA (in the large ribosomal subunit) and presumably not with 16S rRNA in the small subunit (42,51). Nevertheless, mutational evidence has been presented that alteration of 23S rRNA can influence decoding (12,20,38,44).

It is possible that the U70 mutation in $tRNA^{Lys}$ produces its effect on decoding intramolecularly (i.e., through the tRNA molecule to the anticodon region). Because such "communication" between acceptor stem and anticodon region seems to operate for tRNA recognition by some aminoacyl-tRNA synthetases [for review, see (28)], it may be involved similarly in decoding. The possibility that such communication can be achieved in the case of $tRNA^{Lys}$ is perhaps particularly likely because that tRNA may be capable of forming several conformers (65). Several considerations, however, suggest the likelihood of an intermolecular avenue of action, specifically through interaction with ribosomal RNA. It has been shown, first, that a Watson-Crick G-C pair between G2252 in a conserved hairpin loop of 23S rRNA and C74 at the acceptor end of tRNA is required for proper functional interaction of the CCA end of tRNA with the ribosomal P site (51) and, second, that mutants of a tRNA altered in the CCA sequence can lead to altered decoding properties (45). Consequently, the frameshifting caused by the U70 mutant $tRNA^{Lys}$ can be imagined to occur roughly as follows: the formation of the G3·U70 base pair changes the structure of the acceptor stem helix; the altered structure interferes with the proper interaction between the CCA of tRNA and the peptidyltransferase region of 23S rRNA, which in turn leads to abberant translocation involving the anticodon region of the tRNA. This fits the notion proposed by Noller and colleagues (41,42) that tRNA contacts the ribosome with only two of its parts, namely the anticodon region and the acceptor end. Furthermore, such an interaction

between the two ends of tRNA^{Gln} through the glutaminyl-tRNA synthetase molecule in tRNA-dependent aminoacylation has been demonstrated by Söll and coworkers (16,18). However, it is still possible that U70 alters an as yet undiscovered interaction directly with rRNA or that it influences the interaction with 23S or 16S rRNA of some other part of tRNA. It has recently been shown that three other nucleotides (8, 20:1, and 47), which are located in the central fold region of tRNA, can be crosslinked to specific nucleotides of 23S and 16S rRNA (47,49). A computer modeling study has implicated nucleotide 47 in a structural feature that may be necessary for proper binding to the ribosome (60). Finally, Hill and coworkers, using "molecular cutters" covalently linked to residues throughout tRNA, have identified several specific sites of rRNA cleaved after binding of tRNA to the ribosome (5,10).

To shed further light on the U70 decoding effect in *lysT*(U70) tRNA, particularly as to whether the mechanism is intramolecular, intermolecular, or both, it will be helpful to make further mutations in the new *lysT* suppressors. It will also be worthwhile to select or screen for mutations in other translational macromolecules that might reverse or in some other way change the decoding properties of the new suppressors. Likely candidates would be: a) elongation factor G, which is required for translocation; b) elongation factor Tu, mutant forms of which can suppress frameshift mutations; and c) ribosomal RNAs of the small and large subunits. Selections for rRNA mutants correlative to specific tRNA mutants, such as *lysT*(U70) and its derivatives, should be very informative.

It is likely that the influence of a single nucleotide on both tRNA identity and decoding bears some relationship to the coevolution of tRNAs, aminoacyl-tRNA synthetases, and ribosomes [for further discussion of evolutionary aspects, see (26,41,50,54,57)]. However, regardless of the mechanism of altered decoding by U70, it is clear that tRNA^{Lys} "knows" how to make ends meet!

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