The bases of the tRNA anticodon loop are independent by genetic criteria

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ABSTRACT

We employed two methods to study the translational role of interactions between anticodon loop nucleotides. Starting with a set of previously constructed weakly-suppressing anticodon loop mutants of Su7, we searched for second-site revertants that increase amber suppressor efficiency. Though hundreds of revertants were characterized, no second-site revertants were found in the anticodon loop. Second site reversion was detected in the D-stem, thereby demonstrating the efficacy of the search method. As a second method for detecting interactions, we used site-directed mutagenesis to construct multiple mutations in the anticodon loop. These multiple mutants are very weak suppressors and have translational activities that are equal to or lower than that predicted for the independent action of single mutations. We conclude that although the anticodon loop sequence of Su7 has an optimal structure for the translation of amber codons, we find no evidence that interactions between loop bases can enhance translational efficiency.

INTRODUCTION

Inspection of *E. coli* tRNA sequences shows a correlation between the 3' base of the anticodon (or the 5' base of the tRNAs cognate codon), and the sequence that surrounds the anticodon (1). Site-directed saturation mutagenesis of the anticodon stem and loop of the strong amber (UAG) suppressor Su7 (2,3,4) suggests a functional basis for this correlation. Suppression efficiency in Su7, derived from tRNA^{Trp}(UGG), is lowered by substitutions that conflict with the consensus anticodon arm sequence for tRNAs that translate codons beginning with U.

Conversely, a suppressor derived from tRNA^{Glu} is a very weak amber suppressor (5). tRNA^{Glu} reads GAA/G; its anticodon loop sequence agrees with that of other tRNAs that read codons beginning with G, and so disagrees with that of tRNAs that read codons which begin with U. Substitutions in the anticodon stem and loop that increase agreement with the consensus sequence for UNN-reading tRNAs increase suppression efficiency (6) up to 230-fold.

We are interested in discovering the structural basis for these constraints on the

sequence of the nucleotides surrounding the anticodon. One hypothesis is that these nucleotides are constrained by the necessity of forming a higher-order structure that is especially suitable for translating a given (set of) codon(s). Since loop and stem nucleotides are closely apposed (7), it is plausible that they interact through non-covalent forces to create such a structure.

We used a set of previously constructed (2, 3, 4) anticodon loop mutants to probe for functionally important higher-order structures in the anticodon loop. If a given mutant is defective because of the disruption (or creation) of an interaction between two bases within the anticodon loop, a second mutation within the loop that makes a complementary change should partially restore function. Accordingly, a search was made for second-site revertants of seven different single-base substitutions in the anticodon loop of Su7. In addition, we created a set of tRNAs with sequences that differ from that of Su7 at two or three sites in the anticodon loop, in order to observe the effects of multiple mutations without selection for function.

MATERIALS AND METHODS

Bacteria , bacteriophage and plasmids

All bacteria used are derivatives of *E. coli* K12, and are described in Table 1.

Bacteriophage M13 derivatives mp8, mp9, mp10, and mp11 were used as vectors in mutageneses and for sequencing. A 265 bp EcoRI fragment that contains the *aspT* gene and the various amber suppressor derivatives of the *trpT* gene (3) was cloned into the EcoRI site of pOP203.3 (8) for measurement of amber suppressor activity

Plasmids DS281 and DS291 are derivatives of pJC27 (9), which contains a full-length *lacZ* gene transcribed by a UV5 promoter, a phage f1 and a p15a origin, and a chloramphenicol resistance gene. Double-stranded synthetic oligonucleotides with BamHI and HindIII sticky ends were ligated into BamHI, HindIII-cut DNA to create isogenetic *lacZ*⁺ and *lacZ* (Am) alleles. The original sequence of codons 7-10, GCT GCA GGT CGA, was changed to GCT TGG TGG CGA in pDS281, and to GCT TAG TGG CGA in pDS291.

Chemicals , media , and growth conditions

LB and YT (10) were used as rich media for routine cell and phage growth. The E medium of Vogel and Bonner (11) supplemented with 0.2% glucose and 2 µg/ml vitamin B₁ was used as a minimal medium. Phage were routinely plated in H top agar (10). Bacteria and phage were grown at 37 degrees.

Su⁺ revertants were selected on M63 minimal lactose plates (10) in a layer of F top agar (10) which was seeded with mid-log phase MY613, and supplemented with

TABLE 1 E. coli K12 strains used in this work

Strain	Genotype	Origin
JM103recA	Δ lac-pro, thi, strA, endA, sbc15, sbc15, hsdR4, supE, recA56, srl300::Tn10/ F' traD36, proAB ⁺ , lacI ^q , lacZ Δ M15	D. Bradley
PK1216	relB, argA, trp ⁻ , man ⁻ , supE, str ^r , hisG::Tn10, recA ⁻ , / F' 621 man ⁺ , zdd::Tn9	P. Kuempel
PK1315	Hfr PO45, recA56, srl300::Tn10, thr300, relA1, ilv318, spoT1, thi1, rpsE2300	P. Kuempel
LS918	lacZ1000(Am), trp(Am), cys235(Am), str ^r	L. Soll
LS1274	mutD, galU, strA, azi ^r	L. Soll
MY514	recA56, his29(Am), trpA9605(Am), trpR	ref. 4
MY515	recA56, his29(Am), trpR	ref. 4
MY613	lacZ1000(Am), trp(Am), cys235(Am), str ^r , rif ^r , / F' 621 man ⁺ , zdd230::Tn9	This work
MY649	ara, thi, Δ lac-pro, recA56	ref. 9
MY700	mutD, recA56, srl300::Tn10, galU, strA azi ^r , / F' 621 man ⁺ , zdd230::Tn9	This work

0.2% lactose, 0.02% glucose, tryptophan (50 μ g/ml), cysteine (50 μ g/ml), chloramphenicol (30 μ g/ml), X-gal (40 μ g/ml), and IPTG (1mM).

Determination of suppressor efficiency

trpA and trpB gene product activities were assayed in sonic lysates from a trpA9605 (Am) strain (MY514) that also carried the suppressor of interest, and from an isogenic trpA⁺ strain by the method of Smith and Yanofsky (12). The TrpA/TrpB ratio of the nonsense mutant strain was divided by the same ratio of the wild-type strain to give the fraction of normal TrpA levels produced by readthrough of the UAG codon. This system is described more fully elsewhere (4, 13).

Measurement of β -galactosidase activity in MY649 transformed with a suppressor tRNA plasmid and a LacZ plasmid was determined by the method of Miller (10), except that 0.2 ml 2.5 M Na_2CO_3 , rather than 0.5 ml 1 M, was added to stop the reaction. The pseudo-wild *lacZ* gene on pDS281 produces about 15,000 units of activity; readthrough of the isogenetic *lacZ* amber allele on pDS291 by the plasmid-borne suppressor can thus be accurately determined over a range of four orders of magnitude.

tRNA levels and nucleoside modifications

^{32}P -labelled small RNAs were extracted from plasmid-carrying hosts with phenol, purified by polyacrylamide gel electrophoresis, and hybridized to a cellulose-bound M13 clone complementary to tRNA^{Trp}. tRNA^{Trp} and the plasmid-borne suppressor were resolved by further electrophoresis or chromatography (3).

The resolved tRNAs were reduced to nucleotides with a combination of RNases, and the nucleotides are separated by 2D TLC (3).

Oligodeoxyribonucleotide synthesis

Oligonucleotides were synthesized by the methods of Mateucci and Carruthers (14) as modified by Beaucage and Carruthers (15). Modifications to these methods and purification procedures are described in Cline *et al.* (3).

At each ambiguous position in the mutagenizing 13-mer oligonucleotide (corresponding to positions 32, 33, 37, and 38 in the tRNA) the growing chains were divided into four equal parts and separate condensation reactions were done using each of the four 5' dimethoxytrityl nucleoside 3' phosphoramidites. The separate condensations were repooled before the next condensation, so that the resultant set of oligonucleotides could contain all 256 different sequences.

The synthetic oligonucleotides used in the construction of pDS281 and pDS291 were synthesized on an Applied Biosystems 380A DNA synthesizer.

Mutagenesis

Site-directed mutagenesis: The set of chemically synthesized 13-base oligonucleotides was used to direct mutagenesis by a method described in Yarus *et al.* (3). The oligonucleotides of ambiguous sequence were ligated into a precise single-stranded gap in an EcoRI fragment containing the Su7 C33 gene. The EcoRI fragment was ligated into an AvaII-resistant derivative of mp8 given to us by Robert Thompson. The sequence of Su7 C33 contains the only AvaII site in this vehicle. (Su7 C33 is Su7 with C substituted for U at position 33; we use the numbering system of reference 16.) Mutations at position 32 or 32 destroy this AvaII site, and thus allow their selection by AvaII resistance as previously described (4).

Chemical mutagenesis: A drop of ethyl methane sulfonate or of saturated solutions of nitrosoguanidine or hydroxylamine was placed in the middle of an LB plate where a high density of M13mp9-derivative phage had been plated on a lawn of JM103 recA. After growth at 37^o, plaques were transferred to the selective minimal lactose plates described above, and phenotypic LacZ⁺ infective centers were isolated.

MutD mutagenesis: MY700 was grown to a density of 5×10^7 in 5 ml LB plus chloramphenicol, M13 derivative phage were added at a multiplicity of infection of 100, and the culture was allowed to grow to saturation.

Selection of Su⁺ revertants

Mutagenized phage were plated at confluent densities (20,000-25,000 per 85 mm plate) on JM103 recA in 3 ml H top agar on an LB plate. A nitrocellulose (Schleicher and Schuell BA85) or paper (Whatman 1) filter circle 70 or 82 mm in diameter was placed on the plate and allowed to adsorb phage. This filter was then transferred to a selective minimal lactose plate containing a lawn of MY613. Growth to visible colonies requires lactose utilization in this lacZ (Am) strain, and therefore amber suppression by the infecting phage. When mp8 or mp9 were used, as in the chemical and in half of the mutD mutageneses, suppression of two amber mutations in the phage that are toxic to the host when not suppressed (17) was also required. Blue colonies appeared after 2-4 days of incubation at 37 degrees. Phenotypic Su⁺ colonies, chronically infected by M13, were restreaked, single plaques were isolated, and phage stocks were grown in YT.

Plaque hybridizations

Hybridization to an oligonucleotide probe was used to determine the number of substitutions in the anticodon loop and stem of the Su7 gene. The probe used in screening the mutageneses was a 13-base synthetic oligonucleotide, fully complementary to the anticodon loop and stem sequence of the Su7 gene. This oligonucleotide was a gift from Laurel Raftery. In screening the site-directed mutagenesis, an 18-base oligonucleotide complementary to Su7 C33 was employed. Phage streaks were adsorbed to nitrocellulose filters and filters were prepared as described by Maniatis *et al.* (18). The hybridization solution contained 5X Denhardt's solution (19), 6X SSC, 0.1% SDS, and 0.1 mg/ml denatured salmon sperm DNA. The filters were swirled in this solution for an hour or more at 37 degrees, and then incubated at 24 degrees. About 0.1 picomole of 5' ³²P-labelled oligonucleotide was hybridized to the filters for an hour or more. The hybridization solution was removed and the filters were washed in 6X SSC plus 0.1% SDS four times for five minutes at 24 degrees. The wet filters were autoradiographed overnight.

Filters were then subjected to a second wash at 37° and re-exposed. Zero, one and two or more mismatches can be reliably classified: perfect 13 or 18-base duplexes are stable at 37°; one base mismatches form duplexes that are stable at 24°, but show reduced hybridization at 37°; hybrids that contain two or more mismatches cannot be detected at 24°.

RESULTS

Recovery of second-site revertants

We asked whether weakly-suppressing single-base substitution mutants could be reverted by additional mutations in the anticodon loop region. The selection is based on the observation that weakly-suppressing tRNAs have an Su^- phenotype in a hyperaccurate ribosome (streptomycin-resistant) strain (MY613, Table 1). We have previously shown (20) that ribosomal constraint of suppressors by streptomycin-resistant ribosomes and tRNA efficiency are highly correlated. Mutations that sufficiently restore tRNA activity can thus be selected by requiring amber suppression for growth in MY613.

M13 derivatives that bear the $Su7$ mutants indicated in Tables 2 and 3 were mutagenized. MY613 was infected with mutagenized phage and suppression of lacZ1000 (Am) was required for growth. Phage that conferred an Su^+ phenotype were screened by oligonucleotide hybridization to determine whether they were true revertants to $Su7$, or retained the original mutation and gained a second mutation responsible for the Su^+ phenotype.

Chemical mutagenesis: Hydroxylamine, nitrosoguanidine, ethyl methane sulfonate and spontaneous mutagenesis of M13mp9 derivative phage resulted on the isolation of

TABLE 2 RESULTS OF CHEMICAL MUTAGENESIS

Mutation	Number of Su^+ revertants analyzed	Number of true revertants	Reversion events
A32	98	69	AT → CG
G32	119	56	GC → CG
A33	53	22	AT → TA
G33	125	86	GC → TA
C37	57	11	CG → AT
G37	172	142	GC → AT
T37	37	11	TA → AT

True revertants to $Su7$ were identified by oligonucleotide hybridization as described in Methods.

between 40 and 180 Su7 true revertants from each of the seven single base substitution mutants listed in Table 2.

Twenty-four revertants that hybridized as expected for a single mismatch in the anticodon loop were sequenced, and found to be of two classes. One class contains tRNAs that carry the original mutation, but no other change within the tRNA gene. We surmise that the Su^+ phenotype is due in these cases to mutations or rearrangements outside the tRNA gene that increase transcription, processing, or copy number of the tRNA gene. Analysis of restriction digests of RF (replicative form) DNA indicated that some mutants of this type had duplicated the tRNA gene.

The other class of mutants had replaced the original anticodon loop mutation with another, more strongly suppressing anticodon loop mutation. For example, G32, a very weak suppressor, was found to mutate to T32, a stronger suppressor that is phenotypically Su^+ in the selection used. Since all of this class of mutants have already been made and characterized (2, 3, 4) they were not further analyzed.

The mutants sought in this mutagenesis were second site mutations within the anticodon loop that improve the amber suppression efficiency of the primary tRNA mutants. No such alterations were found. We believe that the mutagenesis was effective: the G37 to A37 transition frequency was increased at least 100-fold (data not shown), and 254 transversion mutants that restored the Su7 sequence were recovered

TABLE 3 RESULTS OF MUTAGENESIS BY MUTD

Mutation	Number of phage screened	Mismatches found in anticodon loops of Su^+ phage		Calculated reversion rate	Reversion events
		0	1		
A32	60,000	0	22	$< 1.6 \times 10^{-5}$	AT→CG
G32	64,000	8	37	6.3×10^{-5}	GC→CG
A33	64,000	13	4	1.0×10^{-4}	AT→TA
G33	74,000	31	26	2.1×10^{-4}	GC→TA
C37	100,000	5	6	2.5×10^{-5}	CG→AT
G37	64,000	16	11	1.3×10^{-4}	GC→AT
T37	77,000	0	7	$< 1.3 \times 10^{-5}$	TA→AT

The number of mutations in the anticodon loop of phenotypic Su^+ revertants was determined by hybridization to an oligonucleotide fully complementary to the Su7 anticodon loop and stem. Phage with no mismatches are true revertants to Su7, which arose by one of the mutational events listed in the right-most column. The rate of such mutational events refers to the number of mutations per base pair per replication event, calculated as described in the Appendix. The rate of spontaneous reversion for each phage was determined to be less than 7.4×10^{-6} .

TABLE 4 Suppression of *trpA9605*(Am) and *lacZ*(Am) by plasmid-borne amber suppressors

TrpT allele	Mutation	<i>trpA</i> /B (unknown context)	<i>lacZ</i> (am)/ <i>lacZ</i> ⁺ (GCT TAG TGG)
175	none	0.78	0.83
Single mutants			
501	A32	0.09	0.045
502	G32	0.03	nd
503	T32	0.32	0.38
504	A33	0.09	0.02
505	C33	0.33	0.1
506	G33	0.06	0.02
507	C37	0.03	nd
508	G37	0.06	nd
509	T37	0.01	nd
510	C38	0.45	0.16
512	T38	0.21	0.09
Multiple mutants			
183	A24 A33	0.17	0.15
513	A32 C33	0.024	4.7×10^{-3}
514	T32 A33	0.009	2.5×10^{-4}
515	A33 T38	0.013	1.8×10^{-3}
516	A32 C33 T38	0.006	3.4×10^{-5}
517	A33 C38	0.010	7.3×10^{-5}
518	A32 A33	0.007	6.6×10^{-5}

nd, not determined. Numbers are the fraction of times an amber codon is read rather than terminated. *TrpA/B*: data for single mutants are from reference 4. Standard errors are 5-15% of the mean, except for G32 (33%) and T37 (30%). Except for A24 A33, only two measurements of *TrpA/B* ratios were made for the multiple mutants, so the standard error is not known. *LacZ*(Am): All standard errors are less than 10% of the mean, except for T32 A33 (15%). β -galactosidase activity of the psuedo-wild *lacZ* gene carried on pDS281 is 15340 ± 486 units, and does not vary significantly in the presence of different plasmid-borne suppressors. Background readthrough of the *lacZ*(Am) gene carried on pDS291 is 1.3 ± 0.1 units in the presence of plasmid-borne *trpT*⁺. This value was subtracted from readthrough of the weak suppressors before dividing through by the *lacZ*⁺ value.

from the other tRNA genes (Table 2). It seems unlikely that substitutions at a second-site within the anticodon loop can sufficiently improve the function of the primary tRNA mutants to meet our selective criteria.

MutD mutagenesis: Because the chemical mutagens used might yield a restricted class of mutations, it was desirable to use another mutagenesis protocol. **MutD**, a mutation in the ϵ subunit of DNA polymerase III (21), generates transitions and

transversions at a frequency of 10^{-5} to 10^{-7} (22). The mutator activity is greatly enhanced in rich medium (23). Since M13 DNA is replicated by DNA polymerase III (24), phage are mutagenized by a *mutD* host when grown in rich medium.

Other changes from the protocol used in the chemical mutagenesis were made. Transfer RNA genes were recloned into M13mp10, in order to reduce the stringency of the selection. M13mp9 encodes two amber peptides that are toxic to the host when not suppressed (17). M13mp10 became available after one round of *mutD* mutagenesis had been performed. Although we were most interested in finding second-site revertants in the anticodon loop region, we also began to look for distal revertants by sequencing revertants that carried a single mutation in the anticodon loop.

Reversion to Su7, which requires a single base change, was detected by oligonucleotide hybridization. This allowed us to calculate the rate of *mutD*-induced mutagenesis, as described in the Appendix. The results of these calculations are in Table 3.

All tRNA genes were mutagenized twice by growth of phage in MY700; true revertants of all mutants except A32 or T37 were detected. As with the results obtained by chemical mutagenesis, all (save one; see below) of the Su⁺ revertants were either true revertants or rearrangements or mutations outside of the tRNA gene.

Recovery of a second-site revertant: A second-site revertant of M13mp10 Su7 A33 was found in which G24 had mutated to A. This double mutant, weakly Su⁺ in our selection, has a suppression efficiency, depending on the amber sequence at which it is assayed, two to eight-fold greater than the A33 mutant from which it is derived (Table 4).

The increase in suppression efficiency conferred by the A24 mutation is not due to an increase in tRNA levels or aminoacylation, and is not the result of a change in nucleoside modifications. The A24 mutation actually decreases the level of Su7 A33 from 3% to 0.9% of total tRNA. Thin-layer chromatography revealed no difference in nucleotide modification patterns between Su7 A33 and Su7 A24 A33 (not shown). We measured the effect of tRNA aminoacylation on suppressor function by comparing suppression efficiency in our standard strain, MY514, to that in the same strain containing a 25-fold increased level of the Su7 charging enzyme, GlnRS, a method described in Yarus *et al* (4). The results of this experiment (Table 5) show that Su7 A33 and Su7 A24 A33 are equally stimulated by an increase in GlnRS levels, as would be expected if the two tRNAs are charged to a similar extent in the normal host. We conclude that the effect of the A24 mutation is to stimulate the activity of Su7 A33 at the ribosome.

TABLE 5 Effect of high GlnRS levels
on suppression efficiency

	Su7 A33	Su7 A24 A33
High GlnRS	0.24	0.49
Normal GlnRS	0.09	0.17
High/Normal	2.7	2.9

Suppression efficiencies were measured at *trpA9605* (Am) in the presence of the plasmid-borne suppressor, and in the presence (high GlnRS) or absence (normal GlnRS) of pEF281. pEF281 carries the GlnRS gene and increases the level of GlnRS 25-fold (4).

Site-specific mutagenesis

We used an oligonucleotide preparation containing mixed sequences (see Methods) to generate derivatives of Su7 that contain two or more mutations at positions 32, 33, 37, and 38 in the tRNA sequence. This approach allows the selection and study of multiple mutants without regard to phenotype.

One hundred sixty-five plaques whose DNA survived the mutant enrichment by *Ava*II digestion (see Methods) were analyzed. DNA from these plaques was hybridized to an octadecamer oligonucleotide complementary to the parental Su7 C33 anticodon stem and loop region. 31 of the plaques hybridized very weakly to the probe at 24°. A second wash at 37° destabilized hybridization to another 78 plaques. The remaining 56 plaques hybridized with an intensity equal to that of an Su7 C33 control. Dideoxy sequencing of two of these latter plaques confirmed that they contained the parental Su7 C33 sequence. This class of strongly-hybridizing plaques was not further analyzed.

Fifteen plaques that hybridized at 24° but not at 37° were sequenced. Ten of these phage had A33 substituted for C. Two had G at position 33. Since these mutant tRNAs had been previously isolated (2, 3, 4) they were not pursued. The three remaining mutants differed from the C33 sequence at position 32, where A was substituted for C.

Those phage that hybridized unstably to the C33 probe at 24° were sequenced and found to contain two or more mismatches from Su7 C33. Mutant tRNA genes recovered included the sequences A33 T38, T32 A33, A32 C33 T38, and A33 C38. Multiple isolates of all but the A33 C38 mutant were recovered. A further 41 phage from the non-hybridizing class were isolated and sequenced and from these 41 we recovered one new mutant: A32 A33. About one half of the non-hybridizing phage were found to have incurred deletions in the anticodon region of the tRNA gene, associated with small direct repeats in the sequence.

The 265 bp fragments that contain the mutant tRNA genes were recloned into the *lacP* UV5 expression vehicle pOP203.3 for further analysis. These plasmids were introduced by transformation into MY514, which carries the *trpA*^{A9605} (Am) mutation. Amber suppression efficiency was measured as the ratio of *trpA* to *trpB* gene product (Table 4). All of the multiple mutants are weaker than any of the singly mutant suppressors: each additional change from the original sequence further reduces suppression efficiency.

Determination of β -galactosidase activity due to readthrough of a *lacZ* amber gene cloned on a high copy number plasmid is a more sensitive assay for weakly-suppressing tRNAs, and provided a second message context. The results of this assay (Table 4) are in qualitative agreement with the Trp A/B assays: the ranking of the suppressors by efficiency remains largely unchanged. The relative efficiencies of the tRNAs are not affected by message context. The consistently lower level of readthrough in *lacZ* is probably due to the 3' message context, which is expected to be a poor one (25, 26).

The suppression data in the β -galactosidase and Trp A/B assays allow us to ask if the multiple mutations in the anticodon loop interact. Consider the efficiency of amber suppression to be the probability that an amber codon is read by the suppressor rather than terminated. If two mutations in the loop act independently, then the probability of readthrough by the double mutant tRNA should equal the product of the probabilities of readthrough by each of the tRNAs that contain one of the mutations found in the double mutant.

This type of calculation should only be valid for single mutants that are weak suppressors. Readthrough of amber codons is saturable; suppression efficiency cannot exceed 1. We have previously argued (4) that readthrough will increase hyperbolically with increasing suppressor tRNA activity; only weak suppressors, like these, will show a near-linear relationship between readthrough and suppressor activity.

A plot of suppression efficiencies expected by this method versus those observed, using the *lacZ* and the *trpA/B* data, is shown in Figure 2. Multiple mutants often fall near the line predicted for the independent action of the mutations. Two of the double mutants, Su7 A33 C38 and Su7 T32 A33, have efficiencies much lower than expected at both the assay loci. Thus it is likely that the anomalously low efficiencies of these two mutants are general, not context specific, and not due to some experimental artifact. We conclude that in the case of Su7 A33 C38 and Su7 T32 A33, mutation has created some direct or indirect functional interaction that decreases the efficiency of tRNA action.

DISCUSSION

We are interested in learning how the strength and specificity of the codon-anticodon interaction are facilitated by tRNA structure. The nucleotides that surround the anticodon exert a strong effect on the translational efficiency of suppressors derived from tRNA^{Trp} (4), tRNA^{Glu} (6) and tRNA^{Gln} (27) at UAG codons. It is likely that these nucleotides are influential in other tRNAs at other codons. The aim of the work reported here is to further investigate the extended anticodon; to learn if its influence on translation is primarily due to functional interactions within the extended anticodon itself.

We have used a functional assay - determination of suppression efficiency by mutant tRNAs - to probe tRNA structure. The limitation of this approach is that structure must be inferred, rather than demonstrated, from the experimental results. Furthermore, our methods can only detect specific interactions between bases: those that are changed when the interacting bases are changed. Any interactions common to all bases would not be detected. The advantage of our methods is that the results are obtained *in vivo*, and require no further arguments as to their biological relevance.

The data, along with the results of a complementary study (6), lead us to conclude that specific interactions between the bases of the anticodon stem and loop have little influence on tRNA translational efficiency. Although we do not argue against the existence of physical interactions between these bases, we believe that any such interactions must be non-specific, or of minimal functional relevance.

No second-site revertants are found in the loop

We used a genetic technique, second-site reversion analysis of seven primary anticodon loop mutants, to detect interactions between bases of the anticodon loop of Su7. The detection of second-site revertants would be strong evidence of functional interactions between these bases and might also provide clues to the chemical nature of those interactions.

Analysis of 847 revertants produced by varied means of mutagenesis gave no second-site revertants within the anticodon loop. Our selection required only a partial reversion of the loop mutants. Any interaction that substantially increased suppression activity would have been detected, even though full activity was not restored. Nearly all Su7 single substitution mutants in this region have been detected as suppressors (4); many single mutants are phenotypically su^+ in our selection protocol, and were recovered in these experiments as pseudorevertants. Therefore, the failure to find second-site revertants is not due to a strict requirement for only the unmutated sequence.

Figure 1. Cloverleaf and tRNA^{Phe}-like tertiary structures of Su7. Numbers indicate positions changed by mutation.

We have previously described an example of a secondary change that can improve mutant tRNA performance (4). Su7 G38, an extremely poor suppressor, has the potential to form a 5-membered anticodon loop by the formation of a base pair between G38 and C32 (see Figure 1). Suppression efficiency of Su7 G38 is increased more than 10-fold by a second-site mutation of C32 to T, thus changing a G-C pair to a weaker G-U pair, and so favoring the normal seven base anticodon loop over the mutant five base loop. These results show that it is possible for interactions between mutant bases to increase suppressor efficiency to meet our selective criterion.

The recovery of a new second-site revertant outside the anticodon arm during these experiments demonstrates that our procedures detect the desired phenotype. Our failure to find other efficiency-enhancing interactions between the bases of the anticodon loop suggests that such interactions are exceptional, or nonexistent. Our conclusion is that interactions between the bases around the anticodon are probably not of fundamental importance in optimizing translational efficiency.

Su7 A33 is reverted by a mutation in the D-stem

One second-site revertant outside the anticodon region was detected. A mutation in the D-stem that changes G24 to A increases the suppression efficiency of Su7 A33 two-fold at trpA9605 (Am), and eight-fold at lacZ (Am) (Table 4). The A24 mutation is identical to that found by Hirsh in the tRNA^{Trp}-derived UGA suppressor Su9 (28). We have recently shown that this mutation allows Su7, which reads only amber (UAG)

Figure 2. Independence of multiple loop mutations. The line indicates the suppression efficiency expected if loop mutations act independently.

codons, to read ochre (UAA) codons despite a C-A wobble mispair (29).

As with Su7 (29), the effect of the A24 mutation on Su7 A33 is not due to an increase in tRNA levels, a change in nucleoside modifications, or more efficient aminoacylation. Apparently, the A24 mutation alters the action of tRNA^{Trp}, Su7 and Su7 A33 at the ribosome decoding site.

It is not clear whether the A24 mutation specifically modifies the action of the anticodon loop to both promote C-A wobble pairing and to relieve the A33 defect, or if instead A24 generally stimulates tRNA activity. Either explanation, or a combination, is consistent with the existing data on the action of the A24 mutation (see reference 29). Construction of double mutants, now in progress, should show if the translational effect of the A24 D-arm mutation requires certain anticodon loop structures.

Independence of mutations in multiple mutant tRNAs

We used site-directed mutagenesis to construct derivatives of Su7 that contain multiple substitutions. This approach allows us to assess the effects of multiple changes without assumptions about their phenotypes. We find that all multiple mutants are very weak suppressors. This result is consistent with the conclusion that interactions between nucleotides of the loop do not enhance suppressor efficiency, though we did not study a large number of multiple mutants.

Most of the multiple mutants have a suppression efficiency close to that predicted for the independent action of their constituent single mutations. That is, the product of the efficiencies (the probabilities of peptide chain transmission) of single mutant suppressors often predicts the efficiency of multiply mutant suppressors. This result suggests that the negative effects of the mutants are usually independent, and therefore not the result of novel interactions.

Substitutions that increase the efficiency of a suppressor tRNA also act independently. Raftery and Yarus have created a series of nonsense suppressor derivatives of tRNA^{Glu} (6) that increase the efficiency of this very weak suppressor. The effects of multiple changes are additive: they can be predicted as the product of the effects of individual changes. Thus, in suppressors based on tRNA^{Trp} or tRNA^{Glu}, the effects of multiple neighboring substitutions are predicted by the effects of single substitutions, in both lowering or raising suppressor activity. The functional independence of the bases surrounding the anticodon thus may well be a general property of tRNAs.

In our experiments there are two exceptions to the pattern of independence: A33 C38 and T32 A33 both depress suppression efficiency far below the predicted level in both the *trp* A/B and *lacZ* amber suppression assays: 3 to 4-fold at *trpA9605* (Am) and 30 to 40-fold at *lacZ* (Am) (Table 4, Figure 2). This result indicates that a functional interaction between these mutant nucleotides has been created.

There are several potential sources of lowered suppression efficiency: undermodification of tRNA, lowered levels of mature tRNA, poor charging by aminoacyl-tRNA synthetase, or lowered activity of tRNA on the ribosome. Our study of single mutants of Su7 (4) shows no strong correlation between degree of modification and suppression efficiency. Single-base substitutions of the anticodon loop invariably increase tRNA levels, but often result in lowered aminoacylation and ribosomal activity. Usually, the effect on aminoacylation is less than the effect on ribosomal activity. Extrapolating from these previous results, we suggest that the anomalously

low suppression efficiencies caused by A33 C38 and T32 A33 are most likely to be at the levels of aminoacylation and/or ribosomal function.

What is the molecular nature of the interactions? There is no simple pattern in the genetic results. Nearest neighbors often show little evidence of interaction. For instance, A32 A33, which might be expected to generate an exceptionally strong purine-purine stacking interaction in the 5' side of the loop only moderately decreases suppression efficiency below the level predicted for no interaction. A33 C38 and A33 T38 are chemically similar mutant pairs, yet behave differently. A33 T38 has the efficiency predicted for the independent action of its mutations, while A33 C38 is some 40-fold less efficient than predicted.

The behavior of these multiple mutants cannot be explained easily by tRNA structure alone; the other multiple mutants and the single mutant substitutions appear to act autonomously. These results lead us to consider that the effects of the extended anticodon proceed through the ribosome, rather than affecting the codon-anticodon interaction directly through the tRNA structure.

Additivity is not predicted if nucleotide nearest-neighbor interactions are strong, and affect the property measured. But additive effects of the loop nucleotides might be expected if suppression efficiency is sensitive to the gain or loss of favorable contacts between the tRNA and ribosome. Apparently similar additive interactions between macromolecules have been observed for tRNA^{Tyr} and its aminoacyl-tRNA synthetase (30), and RNA polymerase with *lacP* (31). By analogy with these other macromolecular systems, we speculate that the extended anticodon provides a sort of recognition site for the ribosome. If so, the recognition of this site is unusual and interesting in that binding between the tRNA and ribosome must be strongest when the formation of a cognate codon-anticodon pair has occurred.

APPENDIX

Calculation of mutation rates in MutD

Phage growth involves both a logarithmic phase in which replicative form (RF) DNA is replicated, and a linear phase in which single-stranded viral DNA is synthesized. The following calculation neglects the effects of mismatch repair, and makes the assumptions that the mutation rate is very low, and is approximately equal for both strands. The fraction of mutant molecules is

$$kR \sum_{i=1}^n \frac{1}{2^i} \quad (1)$$

where R is the number of RF molecules in the culture, k is the rate of mutations per

base pair per replication event, and i is the number of rounds of replication of RF molecules. If a certain mutation occurs in the first replication event (synthesis of the minus strand) half of the RF molecules in that cell will subsequently carry that mutation. Similarly, if a mutation occurs in the second round of replication ($i = 2$), $\frac{1}{4}$ of the RF molecules will carry that mutation. The average number of RF molecules in an M13 infected cell is 100-200 (32), equal to at least six doublings. If $i \geq 6$ then $\sum_{i=1}^{\infty} \frac{1}{2^i} \approx 1$. This approximation simplifies equation (1) to kR . If the reversion rate is very small, all phage DNA produced from mutant template will also be mutant. The number of mutant phage produced from mutant RF will be

$$\frac{\Phi}{R} \times kR = \Phi k \quad (2)$$

where Φ is the number of phage at the end of phage growth.

The second source of mutant phage is those phage mutated during replication from wild-type RF. If the fraction of mutant RF $\ll 1$, the number of wild-type RF $\approx R$. Since each mutant phage arises by an independent replication event, the number of this class of mutant phage is equal to Φk . The total number of mutant phage (Φ_{im}) produced by mutation during RF replication and during phage DNA synthesis is equal to

$$\Phi k + \Phi k = 2\Phi k \quad (3)$$

The rate of mutation per base pair per replication event is thus

$$\frac{\Phi_{im}}{2\Phi} \quad (4)$$

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