Evolution of a Transfer RNA Gene Through a Point Mutation in the Anticodon

Margaret E. Saks,*† Jeffrey R. Sampson,‡ John Abelson

The transfer RNA (tRNA) multigene family comprises 20 amino acid–accepting groups, many of which contain isoacceptors. The addition of isoacceptors to the tRNA repertoire was critical to establishing the genetic code, yet the origin of isoacceptors remains largely unexplored. A model of tRNA evolution, termed "tRNA gene recruitment," was formulated. It proposes that a tRNA gene can be recruited from one isoaccepting group to another by a point mutation that concurrently changes tRNA amino acid identity and messenger RNA coupling capacity. A test of the model showed that an *Escherichia coli* strain, in which the essential $tRNA^{Thr}_{UGU}$ gene was inactivated, was rendered viable when a $tRNA^{Arg}$ with a point mutation that changed its anticodon from UCU to UGU (threonine) was expressed. Insertion of threonine at threonine codons by the "recruited" $tRNA^{Arg}$ was corroborated by in vitro aminoacylation assays showing that its specificity had been changed from arginine to threonine. Therefore, the recruitment model may account for the evolution of some tRNA genes.

Transfer RNA participates in two functionally related but physically decoupled biochemical events that are crucial to protein synthesis. The tRNA amino acid–accepting activity (identity) is determined by the aminoacyl-tRNA synthetases, which specifically recognize and aminoacylate the cognate tRNA or tRNAs on the basis of sequence and structural elements (*1–5*). The tRNA amino acid–donating activity occurs at the ribosome after the three nucleotides of the tRNA anticodon engage in complementary base pairing with messenger RNA (mRNA) codons. Cumulatively, these two biochemical events determine the sequence of each protein. Thus, to ensure the fidelity of translation, the correspondence between a tRNA's amino acid identity and its anticodon must recapitulate the genetic code.

Given the central role of tRNA in translation and the near universality of the genetic code, it seems likely that tRNA genes were among the earliest genes to arise (*6*). Efforts to elucidate the events leading to the evolution of tRNA have focused on deducing (i) the sequence and structure of the primordial RNAs that may have originally carried amino acids (*7, 8*), (ii) the molecular events that account for the transition of these RNAs into an RNA having the prototypical tRNA structure (*8, 9*), and (iii) the sequence of the common ancestor of present-day tRNAs (*6, 10*).

In extant organisms, tRNA comprises a multigene family that can be partitioned using the criteria of amino acid specificity, such that each tRNA falls into only 1 of 20 possible amino acid–accepting groups. In turn, several of these groups comprise isoacceptors (tRNAs that accept the same amino acid but have different mRNA codon selectivities). A reasonable a priori assumption is that isoacceptors within each group arose by gene duplication from a common ancestor having the same amino acid identity. However, although this seems to be true in some cases, patterns of sequence similarity that might reflect other evolutionary scenarios have also been observed (*10, 11*). None of these possible scenarios has yet been examined empirically.

One powerful approach to molecular evolution is the use of phylogenetic analysis to deduce the sequence of ancestral genes coupled with the use of biochemical techniques to test their function (*12*). We developed a different but related approach to investigate the molecular mechanisms that account for the evolution of isoacceptors. In this case, sequence (*13*) and functional (*1–5*) information on tRNA was used to formulate a hypothesis about previous historical events, and the hypothesis was tested in vivo using *E. coli* as a model system. The results show that isoacceptors can evolve by gene recruitment events whereby a point mutation in the anticodon trimer recruits a tRNA from one isoaccepting group to another, provided that the synthetase corresponding to the new amino acid already exists.

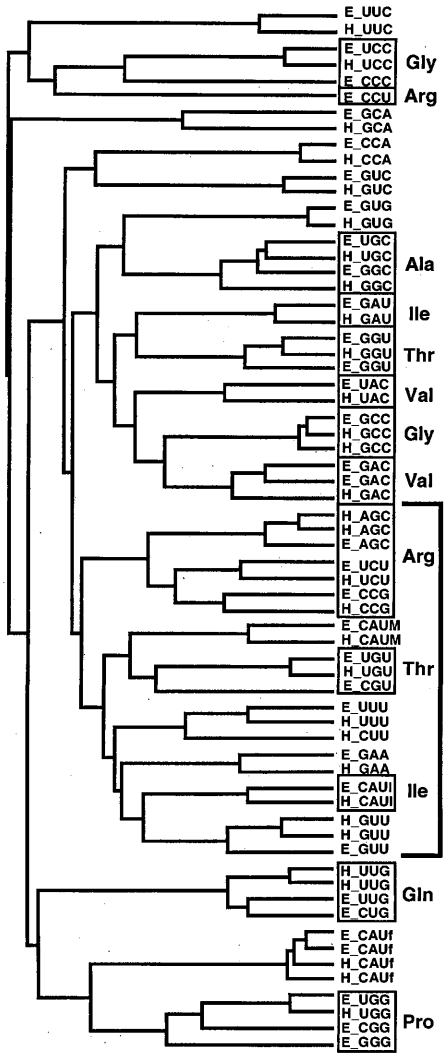

Fig. 1. Dendrogram showing similarities among tRNA gene sequences from *E. coli* (*13, 14*) and *H. influenzae* (*15*). Genes are named by anticodon and species (E, *E. coli*; H, *H. influenzae*). Isoaccepting groups discussed in the text are indicated by the amino acid they accept. The bracket encompasses a cluster that primarily consists of tRNAs having a uridine at position 36 of the anticodon. The dendrogram was generated from aligned sequences (*16*) with the Pileup algorithm [Genetics Computer Group package (*44*)]. Before the analysis, the three anticodon nucleotides were removed from each tRNA so that the resulting clusters would not be influenced by the current decoding capacity of each tRNA.

Models of tRNA gene evolution. Relations among tRNA genes can be explored now that the complete sets of *E. coli* and *Haemophilus influenzae* tRNA gene sequences are available (*14–16*). The similarities among type I tRNA genes from *E. coli* and *H. influenzae* are shown in Fig. 1 (*17*). Of the eight isoaccepting groups in *E. coli* with multiple isoacceptors, three of these groups

Division of Biology 147-75, California Institute of Technology, Pasadena, CA 91125, USA.

*To whom correspondence should be addressed. E-mail: peggy@seqaxp.bio.caltech.edu
†Present address: Department of Biology, University of Oregon, Eugene, OR 97403, USA.
‡Present address: Hewlett-Packard Laboratories, 3500 Deer Creek Road, Mail Stop 25U-5, Palo Alto, CA 94304, USA.

(Ala, Gln, and Pro) form discrete clusters within the dendrogram. In contrast, the isoacceptors within the five other groups (Gly, Arg, Ile, Thr, and Val) are dispersed throughout the dendrogram. This dispersion contradicts a classical model of tRNA gene evolution in which isoacceptors are derived from a single ancestral tRNA having the corresponding amino acid identity. Thus, the mechanisms that account for it are of particular interest. One possibility is that neutral mutations have accumulated since these isoaccepting groups were established. However, this does not seem to be the case because there is relatively little divergence between the *E. coli* and *H. influenzae* genes that encode the same isoacceptor, even though these two species must have diverged more than 160 million years ago (*18*). Alternatively, tRNA genes with the same amino acid identity may be derived from different ancestors. This explanation is consistent with our sequence analyses and has been proposed by others (*10*, *11*) to explain unexpected relations among tRNA genes.

A possible mechanism whereby tRNAs with the same amino acid identity could evolve from different ancestral genes is suggested by relations among tRNAs (Fig. 1). One of the deeply branched clusters in the dendrogram primarily comprises tRNAs that have different amino acid identities and differ at the middle position of the anticodon (position 35), but share a uridine at the 3′ position of the anticodon (U36). Although tRNA affinities in the deeply branched clusters could not be established with a high degree of certainty (*19*), similar patterns have been observed in phylogenetic analyses of archaeal tRNAs (*20*) and in other types of sequence comparisons (*10*, *11*). Together, these observations raise the possibility that amino acid assignments evolved by anticodon mutations.

X-ray diffraction analyses of tRNA-synthetase complexes reveal that the molecular interface at the anticodon can involve a network of interrelated contacts (*21*). That these contacts are sufficient to redirect the amino acid specificity of full-length tRNAs is evidenced by in vitro studies involving anticodon "transplants" (*22–24*) as well as by in vivo studies showing that the amino acid inserted at a single engineered codon in a reporter gene can be dictated by the tRNA anticodon sequence (*5*, *25–27*). We reasoned that because the anticodon can dictate tRNA amino acid specificity as well as mRNA coupling, a tRNA gene could be recruited from one isoaccepting group to another by an anticodon mutation that decreased interactions with the cognate synthetase and promoted interactions with the synthetase corresponding to the new anticodon trimer. As a consequence of concurrently changing tRNA identity and mRNA coupling capacity, a single mutation could potentially result in a tRNA that would be competent to correctly translate a new set of codons in all of the essential endogenous mRNAs. Thus, an anticodon mutation might recruit a tRNA gene from one isoaccepting group to another. Because such a phenomenon offers a possible explanation for the patterns observed in sequence analyses, we explicitly tested the recruitment hypothesis in vivo, using *E. coli* as a model system.

Test of the recruitment hypothesis. The original approach for studying tRNA in vivo identity was not adequate for testing the recruitment hypothesis because it required the tRNA anticodon to correspond to an engineered nonsense (termination) codon in a reporter gene (*28*). An alternative method that could accommodate tRNAs with normal anticodon trimer sequences was subsequently developed (*27*). However, in this method as well as in the original method, tRNA function is assessed with respect to the translation of only a single codon in a reporter gene. Moreover, reporter genes are typically not essential, and the sites used to screen for tRNA amino acid identity usually accommodate a variety of amino acids. Thus, in these reporter systems, there is no inherent cost to cell growth when amino acid misincorporation occurs.

To test the recruitment hypothesis, we used chromosomal manipulation (*29*, *30*) to develop a system for stringently evaluating the in vivo function of a "recruited" tRNA (Fig. 2). We met this criterion by using homologous recombination to create an *E. coli* strain in which the essential, single-copy chromosomal gene for $tRNA^{Thr}_{UGU}$ (*31*) was inactivated and in which the wild-type copy of the gene was simultaneously transferred to the plasmid pMAK705 having a temperature-sensitive replicon (*29*). The chromosomal $tRNA^{Thr}_{UGU}$ gene was inactivated by replacing its anticodon stem and loop with a sequence corresponding to a Drd I restriction endonuclease site (*32*). This altered the normal anticodon stem-loop structure and created a stem consisting of six base pairs closed by a tetraloop in the RNA product. Consequently, this product ($tRNA^{Thr\text{-}Drd\,I}$) was one nucleotide shorter than the wild-type $tRNA^{Thr}_{UGU}$. The knockout strain was transformed with the plasmid p177 bearing the test tRNAs (*33*) and the cells were grown at the nonpermissive temperature to cure them of pMAK705 bearing the wild-type $tRNA^{Thr}_{UGU}$ gene. Thus, after this point, cell viability depended on the activity of the tRNA expressed from p177.

Several factors dictated the choice of tRNAs for testing the recruitment hypothesis (Fig. 3). We chose to knock out the function of the $tRNA^{Thr}_{UGU}$ gene because it is an essential, single-copy gene whose chromosomal location is known (*14*, *15*). Moreover, its manipulation was facilitated by the availability of DNA sequence for the flanking regions (*15*). The decision to attempt to recruit the $tRNA^{Arg}_{UCU}$ gene to function as a $tRNA^{Thr}_{UGU}$ isoacceptor was based on several observations that suggested that these two genes may have been involved in a previous recruitment event. First, the anticodons of the encoded tRNAs differ by only the single nucleotide at the central position (position 35) of the anticodon. Second, our analyses revealed that the sequence of $tRNA^{Thr}_{UGU}$ was as similar to $tRNA^{Arg}_{UCU}$ (68%) as it was to the two other threonine isoacceptors (58% and 68%, respectively). We also reasoned, on the basis of the results of several in vitro aminoacylation studies, that a UCU → UGU change in the $tRNA^{Arg}$ anticodon (a C35G mutation) would decrease aminoacylation by arginyl synthetase [ArgRS (*23*, *34*, *35*)] and would promote recognition by threonyl synthetase [ThrRS (*24*, *36*)] while simultaneously enabling the tRNA to decode the corresponding threonine (ACA and perhaps ACG) codons.

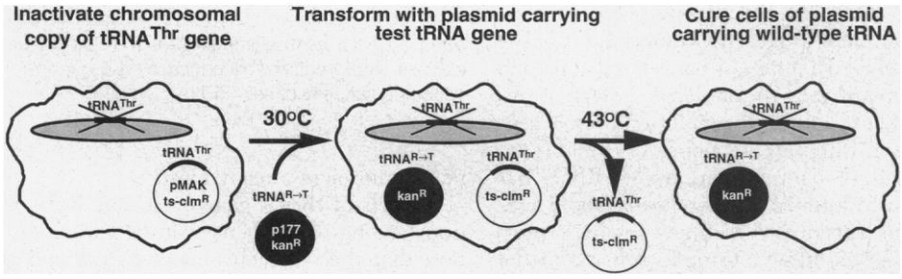

Fig. 2. Scheme for testing the recruitment hypothesis. An *E. coli* knockout strain was created in which the chromosomal copy of $tRNA^{Thr}_{UGU}$ was inactivated and the wild-type copy was expressed from the plasmid pMAK705 having a temperature-sensitive replicon (*29*, *32*). The test tRNAs were cloned into p177 (*33*) and then transformed into the knockout strain. Subsequent growth at 43°C cured the cells of pMAK705 bearing the wild-type copy of $tRNA^{Thr}_{UGU}$ so that the function of the test tRNAs could be evaluated.

However, the possibility existed that the tRNA would retain some of its arginine identity because another ArgRS identity element, A20 (26), remained intact. Consequently, we created two versions of the recruited tRNA: $tRNA^{R\rightarrow T(A20)}$, with only the C35G mutation, and $tRNA^{R\rightarrow T(U20)}$, which also had an A20U mutation (Fig. 3).

Gain of function by a single nucleotide substitution in the tRNA anticodon. Figure 4 shows the growth of the engineered *E. coli* strains under various conditions. In each strain, the chromosomal copy of the $tRNA^{Thr}_{UGU}$ gene is inactivated. At the outset, all strains have the plasmid pMAK705 bearing the wild-type copy of $tRNA^{Thr}_{UGU}$. This plasmid confers chloramphenicol resistance and has a temperature-sensitive replicon. The strains also have the plasmid p177 from which the test tRNAs are expressed and which confers kanamycin resistance. Consequently, all strains can grow at the permissive temperature (30°C) on LB plates containing chloramphenicol or kanamycin. Growth at 43°C blocks replication of pMAK705 and thus causes the associated wild-type copy of the $tRNA^{Thr}_{UGU}$ gene to be lost. This is evidenced by the temperature-dependent chloramphenicol susceptibility of all strains. However, strains will grow after being cured of pMAK705-$tRNA^{Thr}_{UGU}$ if the tRNA expressed from p177 can compensate for the chromosomal knockout.

As expected, strains expressing the two negative control tRNAs ($tRNA^{Arg(A20)}$ and $tRNA^{Arg(U20)}$) were inviable when grown at 43°C in the presence of kanamycin. These tRNAs lack the capacity to couple with threonine codons and thus are unable to compensate for the knockout of the chromosomal copy of the $tRNA^{Thr}_{UGU}$ gene. The inviability of the negative control strains also indicates that mutations that could compensate for the knockout were not arising in the chromosome during the course of the experiment. Strains expressing the positive control ($tRNA^{Thr}_{UGU}$) and either $tRNA^{R\rightarrow T(A20)}$ or $tRNA^{R\rightarrow T(U20)}$ were viable at 43°C in the presence of kanamycin, indicating that these tRNAs compensated for the inactivated chromosomal $tRNA^{Thr}_{UGU}$ gene. The strain expressing $tRNA^{Thr}_{UGU}$ grew somewhat better than those expressing the recruited tRNAs, which suggested that both $tRNA^{R\rightarrow T(A20)}$ and $tRNA^{R\rightarrow T(U20)}$ are not as efficient as the wild-type $tRNA^{Thr}_{UGU}$ at some step in the translation process. However, growth of $tRNA^{R\rightarrow T(A20)}$ and $tRNA^{R\rightarrow T(U20)}$ was comparable, indicating that a single anticodon mutation was sufficient for achieving tRNA recruitment. Hence, these data show that the substitution of a single nucleotide (C35G) in the anticodon of a $tRNA^{Arg}$ results in a tRNA that is competent to insert threonine at threonine codons in all essential endogenous mRNAs to the extent necessary for ensuring cell viability. Thus, a point mutation in the anticodon can recruit a tRNA from one isoaccepting group to another.

Although the results for the negative controls indicated that the new in vivo system was stable during the course of the experiments, we also did a molecular check on the genotype of the strains. Polymerase chain reaction (PCR) amplification with primers specific for the sequence flanking the chromosomal copy of the tuf B operon, followed by restriction digests with Drd I as well as DNA sequencing, revealed that the inactivated $tRNA^{Thr\text{-}Drd\ I}$ gene had not changed during the course of the experiments. Also, the sequences of $tRNA^{Thr}_{UGU}$, $tRNA^{R\rightarrow T(A20)}$, and $tRNA^{R\rightarrow T(U20)}$ (and of their promoters and terminators) were

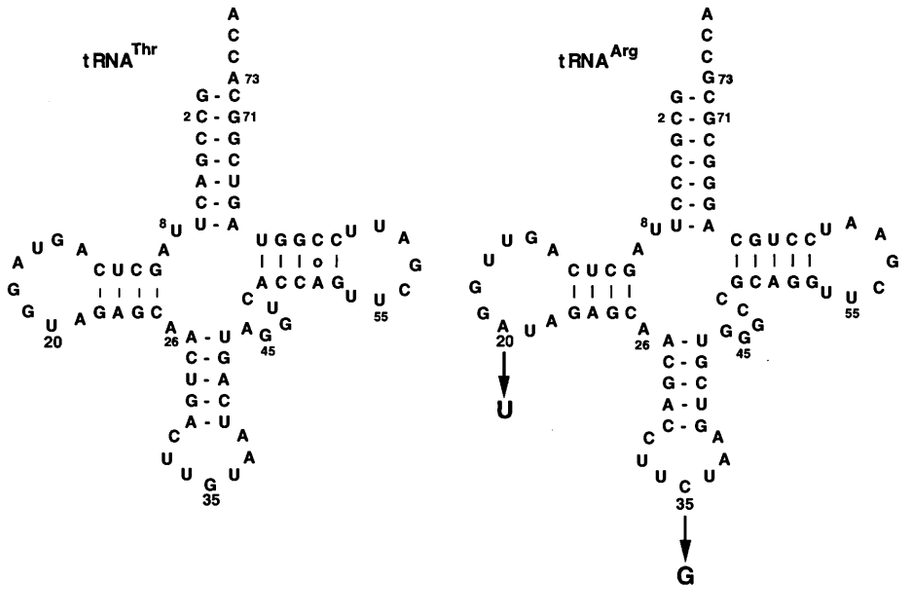

Fig. 3. The sequences of $tRNA^{Thr}_{UGU}$ and $tRNA^{Arg}_{UCU}$ (*13*, *15*). Arrows indicate mutations in $tRNA^{Arg}_{UCU}$ that create the two recruited tRNAs. Both $tRNA^{R\rightarrow T(A20)}$ and $tRNA^{R\rightarrow T(U20)}$ have the C35G mutation, which creates a threonine anticodon (UGU), but they differ with respect to whether nucleotide 20 is A or U.

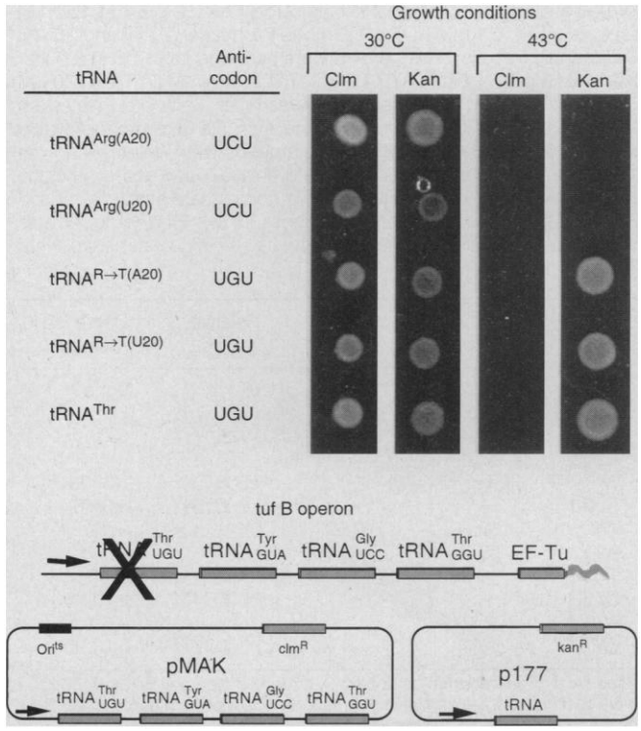

Fig. 4. Growth of *E. coli* strains expressing "recruited" tRNAs. The growth of the two strains expressing the recruited tRNAs ($tRNA^{R\rightarrow T(A20)}$ and $tRNA^{R\rightarrow T(U20)}$) is compared to that of strains expressing the positive control ($tRNA^{Thr}_{UGU}$) and the negative controls ($tRNA^{Arg(A20)}$ and $tRNA^{Arg(U20)}$). The diagram illustrates the genotype of each strain before growth at the nonpermissive temperature. The growth of dilute cell suspensions of each strain (*45*) was tested at 30° and 43°C on LB plates containing either chloramphenicol (Clm) or kanamycin (Kan) at 20 μg/ml.

unchanged after expression from p177 at 43°C in the presence of kanamycin.

Northern blot visualization of tRNA expression (Fig. 5) was used to evaluate how tRNA expression correlates with recruitment. The expression of $tRNA^{Gly}_{UCC}$ provided an internal control for these analyses because it is transcribed from the same operon as $tRNA^{Thr}_{UGU}$ (Fig. 4). Moreover, $tRNA^{Gly}_{UCC}$ and $tRNA^{Thr}_{UGU}$ migrate differently on denaturing polyacrylamide gels and thus can be distinguished in a Northern blot analysis. Lane 1 shows the expression of $tRNA^{Thr}_{UGU}$ and $tRNA^{Gly}_{UCC}$ from the chromosome before engineering the knockout, and lanes 2 to 5 show the appearance of the inactivated $tRNA^{Thr\text{-}Drd\ I}$ product. The increased expression of $tRNA^{Thr}_{UGU}$ and $tRNA^{Gly}_{UCC}$ in lane 2 relative to lane 1 results from the expression of both tRNAs from pMAK705 (Fig. 4). Lanes 3 and 4 show tRNA expression when the $tRNA^{R\rightarrow T(A20)}$ and $tRNA^{R\rightarrow T(U20)}$ strains were grown at 43°C in the presence of kanamycin. Under these conditions, the products of the chromosomally encoded $tRNA^{Thr\text{-}Drd\ I}$ and $tRNA^{Gly}_{UCC}$ genes were detectable, whereas the product of the pMAK705-encoded $tRNA^{Thr}_{UGU}$ was not. This indicates that the strains were cured of pMAK705-$tRNA^{Thr}_{UGU}$ during the actual recruitment test. Lanes 8 and 9 confirm that both $tRNA^{R\rightarrow T(A20)}$ and $tRNA^{R\rightarrow T(U20)}$ were expressed from p177.

The amount of tRNA expressed from p177 relative to that expressed from the chromosome was estimated from the Northern blot analysis. Using the expression of $tRNA^{Gly}_{UCC}$ as an internal control, a comparison of lanes 1 and 5 indicates that when $tRNA^{Thr}_{UGU}$ is expressed from p177, its expression is only about 2.5 times that normally obtained from the chromosomal $tRNA^{Thr}_{UGU}$ locus. A similar comparison of lanes 6 and 8 and of lanes 6 and 9 indicates that the expression of $tRNA^{R\rightarrow T(A20)}$ and $tRNA^{R\rightarrow T(U20)}$ was about 40 to 55 times that of the chromosomally encoded $tRNA^{Arg}_{UCU}$ gene. This difference, which is greater than that estimated for $tRNA^{Thr}_{UGU}$, could be attributable to natural differences in the amount of expression of $tRNA^{Arg}_{UCU}$ and $tRNA^{Thr}_{UGU}$ from the chromosome rather than to differences in the expression of tRNAs from p177. Indeed, the $tRNA^{Arg}_{UCU}$ isoacceptor is responsible for translating the extremely rare AGA arginine codon (*37*) and is thus expected to be in low abundance in the cell (*38*). Thus, it seems likely that with only a modest (2.5-fold) overexpression, both $tRNA^{R\rightarrow T(A20)}$ and $tRNA^{R\rightarrow T(U20)}$ can assume the role of the natural $tRNA^{Thr}_{UGU}$ isoacceptor.

Aminoacylation assays were undertaken to determine the suitability of each tRNA as a substrate for ArgRS and ThrRS (Table 1). These assays revealed that the efficiency (maximal velocity V_{max} divided by Michaelis constant K_m) of ThrRS aminoacylation of a wild-type $tRNA^{Arg}$ was lower than that of a T7 transcript of $tRNA^{Thr}_{UGU}$ by a factor of more than 6600. In contrast, the C35G mutation improved aminoacylation by ThrRS, such that the efficiency of ThrRS aminoacylation of $tRNA^{R\rightarrow T(A20)}$ was lower than that of T7-$tRNA^{Thr}_{UGU}$ by a factor of only 110. The C35G mutation had the opposite effect on aminoacylation by ArgRS; the efficiency of ArgRS aminoacylation of $tRNA^{R\rightarrow T(A20)}$ was lower than that of a T7 transcript of $tRNA^{Arg}_{UCU}$ by a factor of 2500. The fact that a single mutation had opposing effects on aminoacylation by ArgRS and ThrRS is central to the success of the experiment. Apparently, the window of difference in the aminoacylation specificity of $tRNA^{R\rightarrow T(A20)}$ for ArgRS and ThrRS was sufficient to yield a tRNA that predominantly inserted threonine at threonine codons. $tRNA^{R\rightarrow T(A20)}$ would certainly have been lethal had it inserted arginine at threonine codons at rates greater than the normal error rate (*39*). Thus, although the efficiency of ThrRS aminoacylation of $tRNA^{R\rightarrow T(A20)}$ is somewhat less than that of a wild-type $tRNA^{Thr}$, its single amino acid identity endows it with the key char-

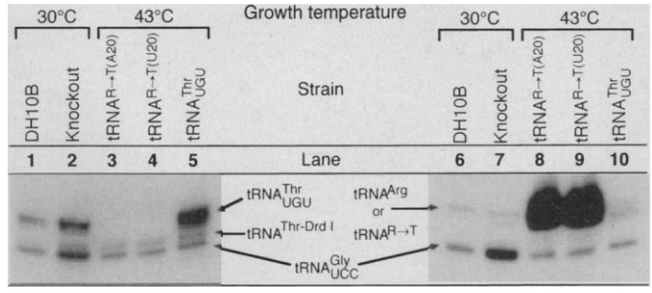

Fig. 5. Northern blots of tRNA expression (*46*). Before nucleic acid isolation, DH10B (the parental strain) and the knockout strain (having the plasmid pMAK705 but not p177) were grown at 30°C. The remaining strains were grown at 43°C so that the amount of tRNA expression, which correlates with recruitment, could be evaluated. Lanes 1 to 5, tRNA expression when the blot was probed with oligonucleotides complementary to $tRNA^{Gly}_{UCC}$, $tRNA^{Thr\text{-}Drd\ I}$, and $tRNA^{Thr}_{UGU}$. Lanes 6 to 10, tRNA expression when the same blot was probed with oligonucleotides complementary to $tRNA^{Gly}_{UCC}$ and $tRNA^{Arg}_{UCU}$. The probe for $tRNA^{Arg}_{UCU}$ also hybridizes to $tRNA^{R\rightarrow T(A20)}$ and $tRNA^{R\rightarrow T(U20)}$. The exposure time for lanes 6 to 10 was about five times that for lanes 1 to 5.

Table 1. Aminoacylation of wild-type and recruited tRNAs by ThrRS and ArgRS (see Fig. 3 for tRNA sequences). All tRNAs were prepared by in vitro transcription with T7 RNA polymerase (*42*). Partially purified *E. coli* ThrRS and ArgRS were used to determine kinetic parameters for each substrate (*43*). Reactions with ThrRS were performed at 37°C in a buffer containing 50 mM tris-Cl (pH 7.5), 8.0 mM $MgCl_2$, 4.0 mM adenosine triphosphate (ATP), 0.1 mM EDTA, 1.0 mM dithiothreitol (DTT), and 20 μM [^{3}H]threonine (specific activity, 10 Ci/mmol). Reactions with ArgRS were performed at 37°C in a buffer containing 50 mM tris-Cl (pH 7.5), 7.0 mM $MgCl_2$, 4.0 mM ATP, 0.1 mM EDTA, 1.0 mM DTT, and 20 μM [^{3}H]arginine (specific activity, 10 Ci/mmol). At each time point, tRNA samples were precipitated on Whatman 3MM paper by washing several times in ice-cold 10% and then 5% trichloroacetic acid followed by a final washing in ice-cold 95% ethyl alcohol. Kinetic parameters were determined from replicated assays that typically used five tRNA concentrations from 0.1 to 9.0 μM and synthetase concentrations from 0.14 to 1.94 U/ml. Assays involving tRNAs and a noncognate synthetase used tRNA concentrations from 5 to 17 μM and synthetase concentrations from 1.16 to 1.94 U/ml. Initial rates were calculated from linear least-squares analyses from five time points. Kinetic parameters were calculated with the program ENZFITTER (Biosoft).

Synthetase	Substrate	Mutation	V_{max} (pmol/min)	K_m (μM)	Relative V_{max}/K_m	Decrease in efficiency*
ThrRS	$tRNA^{Thr}$	WT	26.2	0.42	(1.0)	None
	$tRNA^{R\rightarrow T(A20)}$	C35G	1.64	2.9	0.0091	110
	$tRNA^{R\rightarrow T(U20)}$	C35G A20U	1.42	2.2	0.010	100
	$tRNA^{Arg}$	WT	–	–	<0.00015†	>6600
	$tRNA^{Arg(U20)}$	A20U	–	–	<0.00015†	>6600
ArgRS	$tRNA^{Arg}$	WT	82.3	0.52	(1.0)	None
	$tRNA^{Arg(U20)}$	A20U	66.5	23	0.019	52
	$tRNA^{R\rightarrow T(A20)}$	C35G	0.080	1.3	0.00040	2500
	$tRNA^{R\rightarrow T(U20)}$	C35G A20U	–	–	<0.000077†	>13,000
	$tRNA^{Thr}$	WT	–	–	<0.000077†	>13,000

*The factor by which V_{max}/K_m is lowered for a given substrate compared with the wild-type counterpart. †The reported assay detection limit values were based on the assumption that reliable aminoacylation rates for 10 μM RNA in 1 min corresponded to at least twice the rates obtained in assays performed in the absence of RNA.

acteristic of all tRNAs. An amino acid identity corresponding to the anticodon, rather than aminoacylation efficiency, is likely to be the key prerequisite for the appearance of a tRNA variant in a population. Once such a tRNA appeared, its efficiency of aminoacylation could be improved by the combined forces of mutation and natural selection.

Synthetases typically recognize sets of elements in their cognate tRNAs, and members of these sets often occur in noncognate tRNAs (*3*, *40*). Both $tRNA^{Arg}_{UCU}$ and $tRNA^{Thr}_{UGU}$ have a D-stem comprising four Watson-Crick base pairs and a variable loop comprising five nucleotides (Fig. 3). In addition, they both have a C2-G71 base pair in their acceptor stems. The existence of specificity determinants in the tRNA acceptor stem is well established for many groups (*4*). Indeed, this C2-G71 base pair appears to be important to threonine amino acid identity. It is phylogenetically conserved in threonine tRNAs (*20*), and it comprises one of the $tRNA^{Thr}$ recognition elements (*36*). Thus, it seems likely that sequence overlap is a critical factor that predisposes tRNAs to recruitment into certain isoaccepting groups.

Our results are consistent with the idea that tRNAs within a single isoaccepting group may be derived from different ancestors. As such, they are helpful in interpreting the observed relations among tRNA gene sequences (*10*, *11*, *20*). Because recruitment involves a simultaneous change in tRNA identity and mRNA coupling capacity, tRNA gene recruitment also provides a possible molecular mechanism for Jukes' codon capture hypothesis (*41*).

Recruitment could also play a role in extant organisms once a gene duplication event has occurred. Given the results of the in vitro aminoacylation assays, it seems unlikely that $tRNA^{R\rightarrow T(A20)}$ would replace an active chromosomal copy of $tRNA^{Thr}_{UGU}$. However, a $tRNA^{R\rightarrow T(A20)}$ gene might replace a functionally debilitated copy of an original $tRNA^{Thr}_{UGU}$. Moreover, a $tRNA^{R\rightarrow T(A20)}$ gene might be favored in a population that had not yet evolved a threonine isoacceptor having selectivity for ACA codons. Thus, in addition to a possible role of recruitment in the expansion of tRNA amino acid assignments, recruitment events might be favored when codon usage is in flux.

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31. Transfer RNA genes are named by the three-letter abbreviation for the amino acid that they accept and their three anticodon nucleotides.
32. Four tRNAs and a gene for elongation factor Tu comprise the *E. coli* tuf B operon. A 1-kb fragment of the operon, which encompassed the $tRNA^{Thr}_{UGU}$ gene (nucleotides 40,152 to 41,148 of GenBank accession number U00006), was amplified by PCR and cloned into the Kpn I and Xba I sites of pUC19. The sequence corresponding to the $tRNA^{Thr}_{UGU}$ anticodon stem loop (ACTGACT**TGT**AATCAGT, where underlined sequences form a stem and boldface corresponds to the anticodon) was changed to that of a Drd I restriction endonuclease site (ACGACGGTAACGUCGU) by means of double-stranded mutagenesis (Clontech). The 1-kb fragment containing $tRNA^{Thr\text{-}Drd\ I}$ was then subcloned into the Sac I and Hind III sites of pMAK705. After transformation into *E. coli* strain HS947 [the same as strain DH10B (Gibco-BRL) but with wild-type recA activity], the plasmid recombined with the chromosomal copy of the tuf B operon. Cointegrates were identified by their ability to grow at 43°C on LB plates supplemented with chloramphenicol at 20 μg/ml. The plasmid resolved from the chromosome when cells were grown at 30°C in liquid LB containing chloramphenicol at 20 μg/ml. The strain was stabilized by P1 transduction with *E. coli* strain JC10240 (*Hfr ilv-318 thr-300 thi-1 spc-300 recA56 srlA300::tetA300*) as the donor. The knockout strain having $tRNA^{Thr\text{-}Drd\ I}$ on the chromosome and $tRNA^{Thr}_{UGU}$ transferred to pMAK705 was identified by an inability to grow at 43°C on LB or chloramphenicol plates. The acquisition of *recA56* was confirmed by tests of ultraviolet sensitivity. The strain genotype was also confirmed by PCR amplification of the 1-kb fragment of the tuf B operon using chromosome- and plasmid-specific primers followed by digestion with the restriction endonuclease Drd I (New England BioLabs) and sequencing (USB Sequenase version 2.0) the region of the chromosome encompassing the $tRNA^{Thr\text{-}Drd\ I}$ gene.
33. p177 is a pACYC177 derivative in which the fragment between its BstE II and Bbs I sites has been replaced with a 20-nucleotide linker that recreates the BstE II and Bbs I sites and introduces a single Eag I site. The Eag I–Hinc II fragment was subsequently replaced with a tuf B promoter, followed by the pUC18 polylinker and an rrnC terminator. Thus, in p177, tRNA expression is from the tuf B promoter (nucleotides 40,538 to 40,629 of GenBank accession number U00006) and tRNA expression is terminated by an rrnC terminator [R. A. Young, *J. Biol. Chem.* **254**, 12725 (1979); J. Normanly, R. C. Ogden, S. J. Horvath, J. Abelson, *Nature* **321**, 213 (1986)]. The $tRNA^{Thr}_{UGU}$ gene was obtained by PCR amplification of a portion of the tuf B operon (nucleotides 40,152 to 41,148 of GenBank accession number U00006) followed by digestion with the restriction endonucleases BstU I and Aci I (New England BioLabs). The other tRNA genes were constructed from six overlapping DNA oligonucleotides that included a T7 promoter at the 5′ end and a BstN I restriction endonuclease site at the 3′ end.
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35. Aminoacyl-tRNA synthetases for each amino acid are abbreviated with the standard three-letter amino acid abbreviation followed by RS (for example, argi-

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42. DNA templates were obtained from cloned genes by PCR amplification with Vent polymerase (New England BioLabs) and transcribed with T7-RNA polymerase. The products were gel-purified [J. R. Sampson and M. E. Saks, *Nucleic Acids Res.* **21**, 4467 (1993)].
43. Partially purified *E. coli* ThrRS and ArgRS were obtained from an S100 extract prepared through the DEAE-cellulose chromatographic step [K. H. Muench and P. Berg, in *Procedures in Nucleic Acid Research*, G. L. Cantoni and D. R. Davies, Eds. (Harper & Row, New York, 1966), pp. 375–383]. The specific activities per milligram of protein for ThrRS and ArgRS were 3.4 and 5.7, respectively. One unit is defined as the amount of synthetase required to aminoacylate 1.0 nmol of the cognate tRNA per minute at 5 μM tRNA under the reaction conditions described in Table 1.
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45. The knockout strain was transformed with the relevant p177 constructs and grown on LB/Kan plates at 30°C. Dilute cell suspensions were made from the resulting colonies. A 1-μl sample of each cell suspension (6700 to 19,200 cells) was pipetted onto the test plates and incubated at the appropriate temperature for 24 hours.
46. Total nucleic acid was isolated from each strain [V. B. Chanda, Ed., *Current Protocols in Molecular Biology* (Wiley, New York, 1990)]. From each sample, ~0.5 A_{260} unit was subjected to electrophoresis on 8% denaturing polyacrylamide gels (19:1, 20 cm by 40 cm) and transferred to GeneScreen (New England Nuclear). Probes were γ-^{32}P–end-labeled oligonucleotides specific for nucleotides in the anticodon stem, variable loop, and T Y C-stem of each tRNA: $tRNA^{Gly}_{UCC}$, positions 31 to 56; $tRNA^{Thr}_{UGU}$, positions 38 to 62; $tRNA^{Thr\text{-}Drd\ I}$, positions 24 to 48; $tRNA^{Arg}_{UCU}$, $tRNA^{R\rightarrow T(U20)}$, and $tRNA^{R\rightarrow T(U20)}$, positions 38 to 62. Band intensities were quantified with a Molecular Dynamics PhosphorImager and associated software.
47. We thank S. R. Kushner, V. Maples, and H. Shizuya for providing *E. coli* strains and for guidance in constructing the knockout strain; and A. Zera, F. Gould, and M. Lynch for thoughtful comments. Supported by NIH grant GM 48560.

3 July 1997; accepted 23 January 1998