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tRNA–guanine transglycosylase from *Escherichia coli*: Recognition of noncognate–cognate chimeric tRNA and discovery of a novel recognition site within the TΨC arm of $tRNA^{Phe}$

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ABSTRACT

tRNA–guanine transglycosylase (TGT) is a key enzyme involved in the posttranscriptional modification of tRNA across the three kingdoms of life. In eukaryotes and eubacteria, TGT is involved in the introduction of queuine into the anticodon of the cognate tRNAs. In archaebacteria, TGT is responsible for the introduction of archaeosine into the D-loop of the appropriate tRNAs. The tRNA recognition patterns for the eubacterial (*Escherichia coli*) TGT have been studied. These studies are all consistent with a restricted recognition motif involving a U-G-U sequence in a seven-base loop at the end of a helix. While attempting to investigate the potential of negative recognition elements in noncognate tRNAs via the use of chimeric tRNAs, we have discovered a second recognition site for the *E. coli* TGT in the TΨC arm of in vitro-transcribed yeast $tRNA^{Phe}$. Kinetic analyses of synthetic mutant oligoribonucleotides corresponding to the TΨC arm of the yeast $tRNA^{Phe}$ indicate that the specific site of TGT action is G53 (within a U-G-U sequence at the transition of the TΨC stem into the loop). Posttranscriptional base modifications in $tRNA^{Phe}$ block recognition by TGT, most likely due to a stabilization of the tRNA structure such that G53 is inaccessible to TGT. These results demonstrate that TGT can recognize the U-G-U sequence within a structural context that is different than the canonical U-G-U in the anticodon loop of $tRNA^{Asp}$. Although it is unclear if this second recognition site is physiologically relevant, this does suggest that other RNA species could serve as substrates for TGT in vivo.

Keywords: enzymology; queuine; RNA recognition; transglycosylase; tRNA modification

INTRODUCTION

To date, over 90 naturally occurring modified nucleosides have been identified in RNA, with over 75 of these found in tRNA (Limbach et al., 1994). In a number of cases, enzymes involved in the biosynthesis of these modified bases have been identified and to varying degrees, characterized (Garcia & Goodenough-Lashua, 1998a, 1998b). The exact physiological and biochemical roles of most modified bases in vivo are still largely unknown; however, at least one base modification changes the amino acid identity of the tRNA (Muramatsu et al., 1988a, 1988b). Queuine (Q, 7-(4,5-cis-dihydroxy-1-cyclopenten-3-ylaminomethyl)-7-deazaguanine) is one example of a hypermodified base. It is present only in the wobble position (#34) of the anticodon loop of tRNAs with anticodon sequences of GUN (tRNAs Asn, Asp, His, and Tyr) of most eubacteria and eukaryotes. In higher organisms, queuine-containing tRNA is further modified by a glycosylation of the cyclopentene diol with either mannose or galactose (Kasai et al., 1976).

A key enzyme involved in the incorporation of queuine into tRNA is tRNA–guanine transglycosylase (TGT). Much work has been done to characterize the eubacterial TGT (*Escherichia coli* and *Zymonomas mobilis*) in terms of substrate recognition (Curnow et al., 1993; Curnow & Garcia, 1994, 1995; Nakanishi et al., 1994; Hoops et al., 1995), mechanism (Reuter et al., 1994; Romier et al., 1996b), and three-dimensional structure (Romier et al., 1996a). Results from recent studies (Kung & Garcia, 1998) suggest that there are no primary or secondary structures other than the major determinant for TGT recognition (i.e., the U-G-U sequence within a seven-base loop at the end of a helix) that stand out as important positive recognition elements for the *E. coli*

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TGT. Although there appear to be no queuosine-noncognate tRNAs that contain the U-G-U (in positions 33–35) sequence, there are many tRNAs with either U-G-N or U-N-U sequences. It seems reasonable to speculate that these tRNAs may have a significant degree of affinity for TGT given their similarity to the queuosine-cognate tRNAs. If true, then queuosine-noncognate tRNAs may be inhibitors of TGT in vivo, presumably competitive with respect to tRNA. It is possible that these tRNAs may contain negative recognition elements (i.e., sequences that impair their ability to bind to TGT) that would serve to minimize any interaction they might have with TGT and thereby eliminate any competitive inhibition. One example of such a tRNA is the yeast $tRNA^{Phe}$ (Fig. 1). This tRNA has the sequence U-G-A (33–35) in its anticodon loop.

To determine if there are any TGT negative recognition elements in yeast $tRNA^{Phe}$, a chimeric tRNA was designed and characterized. This tRNA (SCF/D, Fig. 1) is essentially the yeast $tRNA^{Phe}$ with the seven bases of its anticodon loop replaced with the corresponding bases of yeast $tRNA^{Asp}$ [a queuosine-cognate tRNA that we have previously characterized (Kung & Garcia, 1998)]. These specific tRNAs were chosen because they both have been highly characterized. The X-ray crystal structures for both tRNAs have been solved (for yeast $tRNA^{Phe}$: Robertus et al., 1974; Westhof et al., 1988; for yeast $tRNA^{Asp}$: Dumas et al., 1985; Westhof et al., 1985, 1988), and the relationship between various tertiary nucleotides and the correct folding of yeast $tRNA^{Phe}$ has been studied thoroughly (Behlen et al., 1990; Sampson et al., 1990).

During the course of this study, we found that the in vitro-transcribed yeast $tRNA^{Phe}$ [SCF(in vitro)], though not a queuosine-cognate tRNA, is a substrate for *E. coli* TGT. This unexpected discovery led to the investigation of the recognition of SCF(in vitro) by TGT. From this study a recognition motif (within the TΨC arm) different from the one located in the anticodon stem/loop region has been identified. Kinetic analyses (K_m, k_{cat}, and k_{cat}/K_m) of synthetic mutant oligoribonucleotides corresponding to the TΨC arm of the yeast $tRNA^{Phe}$ indicate that the site of TGT action is G53 (within a U-G-U sequence at the transition of the TΨC stem into the loop).

RESULTS

Binding of full-length tRNAs to TGT via native PAGE band shift

Previously we have shown that the *E. coli* TGT exists as a trimer in solution and that it forms a monomer•tRNA complex in the presence of tRNA that is observable by a band shift (to lower M_r) on native PAGE (Curnow & Garcia, 1994; Reuter et al., 1994). Native PAGE band-shift experiments were performed to investigate the gross interaction between TGT and the tRNAs under investigation (Fig. 2). [In these experiments, 3 μM TGT were incubated with 42 μM tRNA (in the absence of guanine) prior to electrophoresis.] The interactions of unmodified yeast $tRNA^{Phe}$ and yeast $tRNA^{Asp}$ with TGT were also studied as controls. As shown in Figure 2, the presence of SCF/D causes the trimeric TGT (lane 1) to dissociate and form a TGT monomer•tRNA complex (lane 4), a phenomenon similar to that which has been observed for queuosine-cognate tRNAs (SCD in lane 2; Kung & Garcia, 1998). The extent of band shifting resulting from the coincubation of SCF/D with TGT is at least similar to or even greater than those observed for the queuine-cognate tRNAs ECY and SCD. These results suggest that the affinity between the hybrid tRNA and TGT is not dramatically impaired by the presence of the "body" of a noncognate tRNA. The band shift for

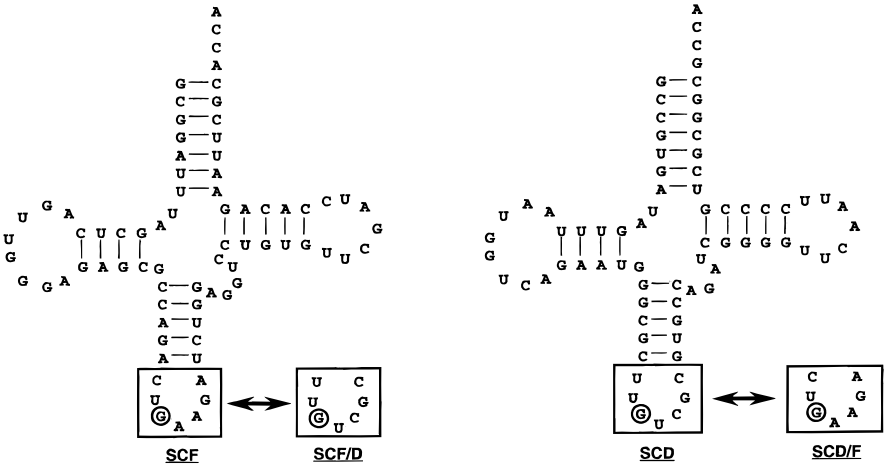

FIGURE 1. Secondary structures of unmodified yeast $tRNA^{Phe}$ [SCF(in vitro)], $tRNA^{Asp}$ (SCD), chimeric $tRNA^{Asp\text{-}Phe}$ (SCD/F), and chimeric $tRNA^{Phe\text{-}Asp}$ (SCF/D). The chimeric tRNAs consist of the body of one tRNA (SCF or SCD) and the anticodon loop of the other tRNA. The exchanged anticodon loops are boxed. G34, the position where the queuosine modification takes place, is circled.

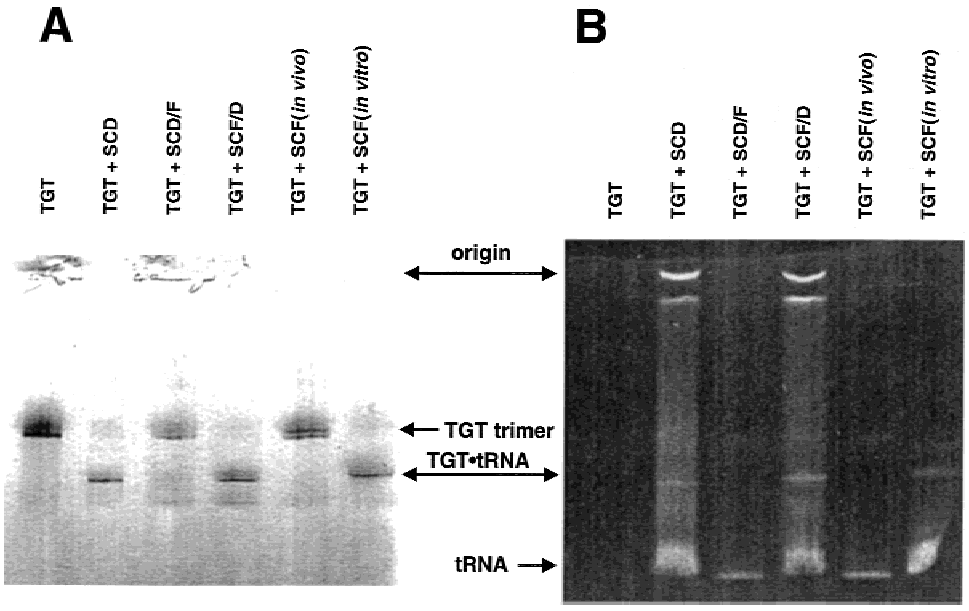

FIGURE 2. Native PAGE of TGT and TGT•tRNA complexes-I. TGT (3 μM) was preincubated with tRNA (42 μM) in 10 mM HEPES, pH 7.5, 10 mM $MgCl_2$, and 1 mM EDTA at 37 °C for 30 min. Approximately 4 μL were loaded in each lane. **A**: The gel stained with Coomassie blue to visualize the protein-containing bands. **B**: The gel stained with ethidium bromide and visualized by UV-transillumination to identify RNA-containing bands. (Note that although the gel images are cropped, all bands on the gels appear in the images.)

SCF/D exhibits a second, faint band at an M_r lower than that for the typical TGT monomer•tRNA band that may be due to a small amount of monomeric TGT present in the incubation. It is difficult to tell from the ethidium-stained gel (Fig. 2B) if there is RNA present in this band. SCD/F, the reverse chimera, exhibits little binding to TGT (Fig. 2, lane 3). Results from the band-shift experiment shown in Figure 2 also reveal that unmodified SCF(in vitro), though not a cognate tRNA for TGT, binds to the enzyme (lane 6).

Formation of a stable complex between full-length tRNAs and TGT

Formation of a TGT•RNA complex that is stable to mild denaturing conditions has been previously observed (Romier et al., 1996b). (This complex is thought to be formed by nucleophilic attack of the enzyme upon the 1′ carbon of G34, displacing guanine, and forming a covalent TGT–RNA intermediate.) The formation of this stable complex was monitored via denaturing-PAGE. A band consistent with a stable complex (~70 kDa) is seen for the queuosine-cognate tRNA SCD (Fig. 3, lane 2). Bands consistent with stable complexes are also seen for SCF/D and SCF(in vitro) (Fig. 3, lanes 4 and 6, respectively). The bands for the stable complexes are fairly faint, perhaps because of a low amount of stable complex formed under these conditions or because of the instability of the TGT•tRNA complex under the denaturing conditions. Indeed we have observed that harsher denaturing conditions (e.g., boiling the samples) totally eliminates the stable complex band. No stable complex bands are observed for SCD/F and SCF(in vivo) (Fig. 3, lanes 3 and 5, respectively), consistent with these tRNAs not being true substrates for TGT.

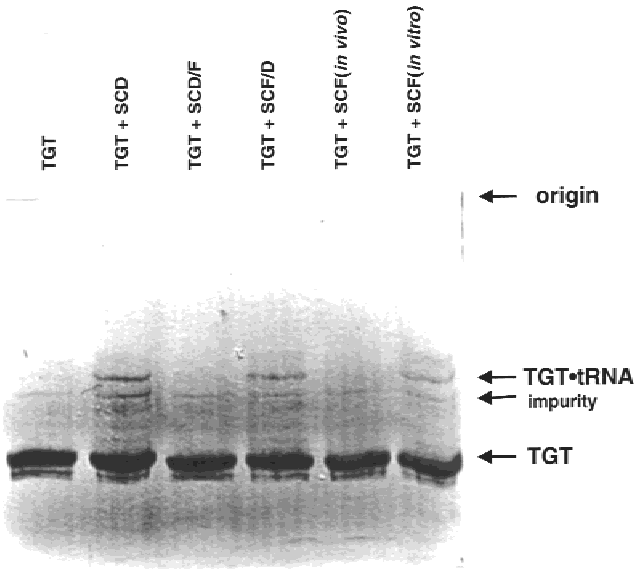

FIGURE 3. Denaturing PAGE of TGT and TGT•tRNA complexes. A 10-μL volume of TGT (3 μM) was preincubated with each tRNA (42 μM) in 10 mM HEPES, pH 7.5, 10 mM $MgCl_2$, and 1 mM EDTA at 37 °C for 1 h. Ten microliters of SDS (5%), β-mercaptoethanol (10%), and bromophenol blue (0.02%) were added to each sample and they were incubated for an additional 1 h at 37 °C. Approximately 4 μL were loaded in each lane. The gels were stained with Coomassie blue to visualize the protein-containing bands.

Kinetic parameters of full-length tRNAs with TGT

Kinetic results for SCF/D (Table 1) indicate that the chimeric $tRNA^{Phe\text{-}Asp}$, containing the queuine-cognate anticodon loop, is a substrate for TGT with kinetic parameters comparable to those for the unmodified cognate tRNAs(Kung & Garcia, 1998). The plots of the initial velocity versus various tRNA concentrations for SCF/D (Fig. 4A) indicate that Michaelis-Menten kinetics are followed for this chimera. Surprisingly, kinetic analyses also show that the unmodified SCF(in vitro) is a substrate for TGT, albeit significantly poorer than the queuosine-cognate tRNAs ECY and SCD (Table 1). The presumably fully modified SCF(in vivo) and the reverse chimera SCD/F had activities that were too low to accurately determine the kinetic parameters.

To investigate the very low level of activity observed for SCF(in vivo) and SCD/F, the relative initial velocities of guanine incorporation were determined for a series of tRNAs at a single concentration of 25 μM (Table 2). The results indicate that the activities for SCF(in vivo) and SCD/F are very low ($\sim$1%) and are roughly equal to the guanine incorporation seen for a totally noncognate tRNA ($tRNA^{Ala}$ from *Archaeoglobus fulgidus*).

Binding of minihelical RNA analogs to TGT via native PAGE band shift

Minihelical RNA analogs corresponding to the TΨC arm of SCF [extended to ensure stability of the helix at the assay temperature of 37 °C (Curnow & Garcia, 1995)] were generated (Fig. 5) via chemical synthesis. The ability of TGT to recognize these minihelical RNAs was determined by native-PAGE band-shift assay as discussed above for the full-length tRNAs. Panel A of Figure 6 shows that all of the minihelical RNAs elicit the characteristic band shift when incubated with TGT. This indicates that all of the minihelical RNAs bind to TGT under the conditions of the assay.

TABLE 1. Kinetic parameters for several tRNA analogs.

Analog	K_m[a,b] (μM)	k_{cat}[a,b] (10^{-3}•s^{-1})	k_{cat}/K_m (10^{-3}•s^{-1}•μM^{-1})	relative k_{cat}/K_m[c]
ECY[d]	3.63 (0.44)	4.92 (0.19)	1.36 (0.22)	1
SCD[d]	1.85 (0.42)	6.74 (0.42)	3.65 (1.06)	2.7
SCF/D	0.36 (0.15)	0.83 (0.09)	2.32 (1.20)	1.7
SCF(in vitro)	8.97 (2.54)	0.73 (0.10)	0.08 (0.03)	0.058
SCF(in vivo)	n/d[e]	n/d	n/d	n/d
SCD/F	n/d	n/d	n/d	n/d

[a] Standard errors are shown in parentheses.
[b] Kinetic parameters are determined from the average of two [ECYMH and SCF(in vitro)], three [ECY and SCFMH(TΨC)], or four (SCF/D) replicate determinations of initial velocity data.
[c] The relative k_{cat}/K_m was calculated relative to that for ECY.
[d] Kinetic parameters for ECY and SCD were taken from Kung & Garcia (1998).
[e] Not determined. The activities for the fully modified SCF(in vivo) and SCD/F were too low to determine kinetic parameters (see Table 2).

Formation of stable complex between minihelical RNA analogs and TGT

Formation of stable complexes between TGT and the minihelical RNA analogs was monitored via denaturing-PAGE as discussed above for the full-length tRNAs. Bands consistent with a stable complex ($\sim$50 kDa) are clearly seen for all of the minihelical RNAs with the exception of SCFMH(TΨC) (G53C) (Fig. 6B, lane 5). Owing to the fact that this stable complex is presumed to be a mechanistic intermediate, this strongly suggests that although SCFMH(TΨC) (G53C) can bind to TGT noncovalently, it is not a substrate. We note that the stable complex bands for the minihelical RNAs are much stronger than those for the full-length tRNAs (Fig. 3). The reason for this is unclear, although it is possible that the smaller minihelical RNAs form a more stable (to the denaturing conditions used) stable complex with TGT than the full-length tRNAs. Alternatively, the proportion of enzyme in the stable complex at any one moment may be higher for the minihelical RNAs. If product release were overall rate-limiting for the TGT reaction, then the smaller k_{cat}s observed for the minihelical RNA versus the full-length RNAs (Tables 1 and 3) would be consistent with a higher population of the covalent intermediate.

Kinetic parameters of minihelical RNA analogs with TGT

Kinetic analyses were also performed on the minihelical RNA analogs (Fig. 7). These results (Fig. 7 & Table 3) reveal that SCFMH(TΨC), SCFMH(TΨC) (G51C), and SCFMH(TΨC) (G57A) are substrates for TGT, with kinetic parameters somewhat poorer than those for a similar oligoribonucleotide (ECYMH) corresponding to the anticodon arm of ECY that we have previously characterized (Curnow & Garcia, 1995). Figure 7 shows that these minihelical RNAs follow Michaelis-Menten kinetics, albeit with relatively large error bars (most likely due to the higher errors associated with lower levels of activity). Consistent with the results of the denaturing-PAGE analysis, SCFMH(TΨC) (G53C) was found to have no detectable activity at concentrations up to 25 μM and incubation times up to 4 h.

Inhibition of TGT by SCFMH(TΨC) (G53C)

Native-PAGE analysis indicated that SCFMH(TΨC) (G53C) can bind to TGT, however it is not a substrate for TGT. This suggests that SCFMH(TΨC) (G53C) should be able to inhibit the TGT reaction (presumably competitive with respect to tRNA). To address this issue,

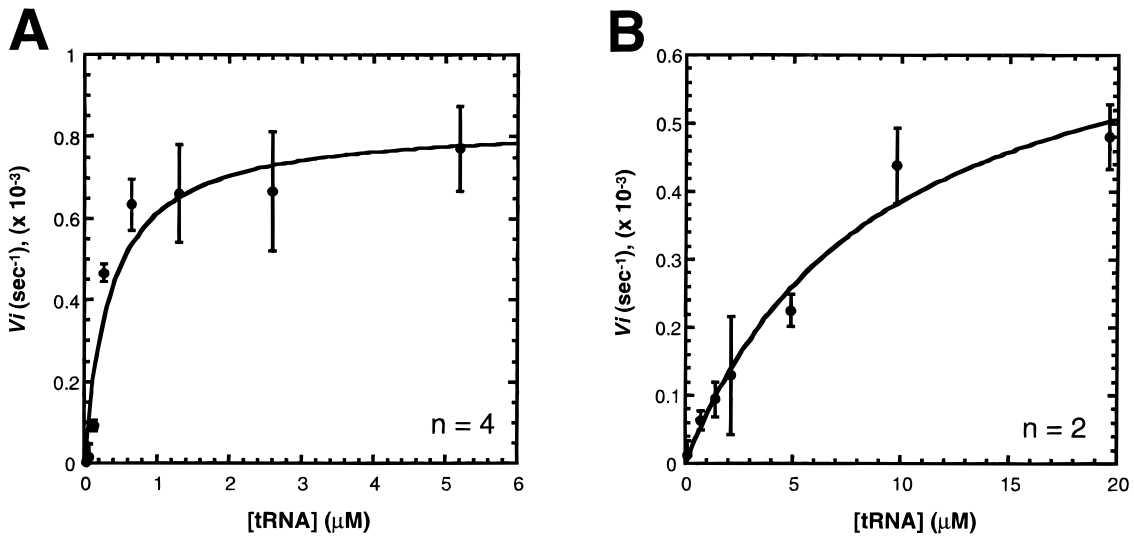

FIGURE 4. Michaelis-Menten analyses of unmodified yeast $tRNA^{Phe\text{-}Asp}$ chimera (SCF/D) and yeast $tRNA^{Phe}$ [SCF(in vitro)]. **A**: SCF/D; **B**: SCF(in vitro). The averages of data points obtained from two or four independent determinations are plotted. The curves represent fits of the data calculated by nonlinear regression. Error bars are generated from the standard deviation within each point.

the ability of SCFMH(TΨC) (G53C) to inhibit the TGT reaction with ECYMH was investigated. For practical reasons (largely due to the quantities of SCFMH(TΨC) (G53C) required) we were not able to determine a true K_i for SCFMH(TΨC) (G53C). However, the IC_{50} for SCFMH(TΨC) (G53C) inhibition of TGT activity with 0.5 μM ECYMH was determined to be $\sim$150 μM (Fig. 8). Assuming that the inhibition mode is competitive with respect to ECYMH, one can estimate K_i for SCFMH(TΨC) (G53C) using the following equation:

$$IC_{50} = K_i([S]/K_m + 1)$$

where [S] = 0.5 μM, and K_m = 4.68 μM (Table 3). This gives an approximate K_i of 135 μM.

TABLE 2. Relative initial velocities of guanine incorporation into tRNAs at 25 μM.

tRNA	Relative initial velocity[a] (%)
ECY	100
SCD	100
SCF/D	3
SCD/F	0.5
AFA[b]	0.3
SCF(in vitro)	7
SCF(in vivo)	1.5

[a]The relative initial velocities were determined under the following conditions: 250 nM TGT, 20 μM guanine, with aliquots taken over time ranges of 0–12 min (ECY) up to 0–4 h (SCD/F).

[b]AFA is an in vitro transcript of the $tRNA^{Ala}$ from *A. fulgidus*. It contains no UGU sequences. This was used as a totally noncognate tRNA controlling for nonspecific TGT activity.

This demonstrates that although SCFMH(TΨC) (G53C) is not a substrate for TGT, it does bind to the enzyme in the same site as (or at least competitively with) the substrate RNAs. This strongly suggests that the inactivity of SCFMH(TΨC) (G53C) is due to the absence of guanine at position 53, indicating that this is the site of TGT action.

DISCUSSION

Our initial goal in this study was to investigate the possibility of the existence of negative recognition elements within queuine-noncognate tRNAs. In our context, negative recognition elements are specific sequence/structural motifs that prevent queuine-noncognate tRNAs from being recognized by TGT. To do this, we constructed a chimeric tRNA consisting of the body of $tRNA^{Phe}$ and the anticodon loop of $tRNA^{Asp}$. Native-PAGE band-shift experiments reveal that this chimeric tRNA (SCF/D) does indeed bind to TGT in a fashion qualitatively similar to that of queuine-cognate tRNAs. Additional band-shift experiments performed under gentle denaturing conditions show that a stable complex is formed between SCF/D and TGT. This stable complex is thought to be a covalent intermediate in the TGT reaction (Grädler et al., 1999; Romier et al., 1996b). A reverse chimeric tRNA consisting of the body of $tRNA^{Asp}$ and the anticodon loop of $tRNA^{Phe}$ was constructed and this tRNA (SCD/F) exhibited little detectable binding to TGT.

In vitro kinetic studies indicate that SCF/D is indeed a substrate for TGT with kinetic parameters similar to those for queuine-cognate tRNAs (ECY & SCD; Kung & Garcia, 1998). The kinetic parameters for SCF/D are

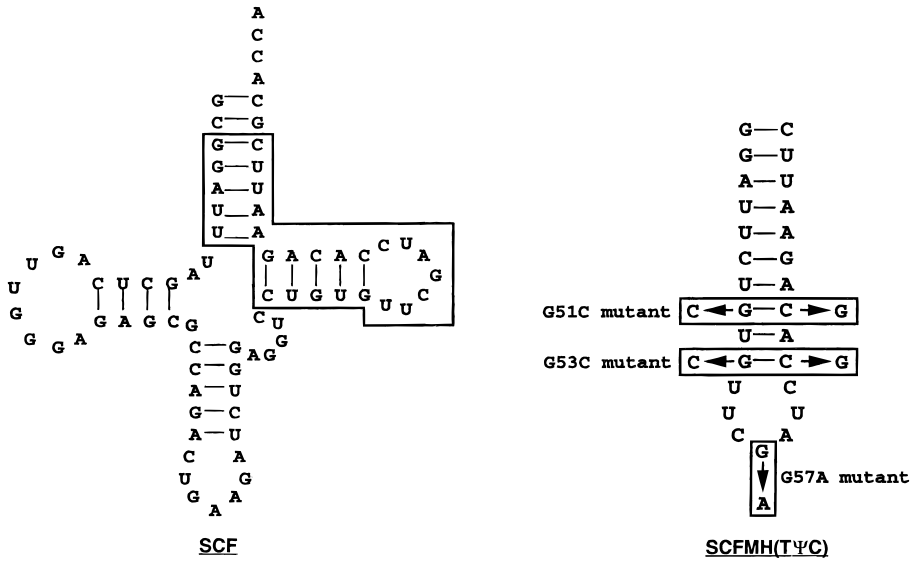

FIGURE 5. Secondary structures of unmodified yeast $tRNA^{Phe}$ [SCF(in vitro)] and its TΨC stem/loop minihelix analog [SCFMH(TΨC)]. Nucleotide sequence (boxed) of SCFMH(TΨC) is taken from the TΨC stem/loop and part of the acceptor stem of SCF(in vitro).

slightly different than those for SCD, both K_m and k_{cat} are lower. One possible explanation for this is that the chimeric tRNA may bind tighter to the enzyme. This implies a higher binding energy and thus a lower dissociation constant for the chimeric tRNA. The stabilization of the TGT•tRNA complex in the ground state may result in a larger activation energy for the reaction if the transition state is not stabilized equivalently. This would yield a smaller k_{cat} because of a larger energy barrier. It has been proposed that a conformational change in the anticodon loop may be necessary for G34 to reach the TGT active site (Romier et al., 1996a). If indeed, SCF/D does bind TGT more tightly in the ground state, this proposed conformational change could be impeded. This then would provide a molecular mechanism for the increased energy barrier and reduced k_{cat}. Alternatively, because the TGT assay used to kinetically evaluate the tRNAs is a guanine-for-guanine ex-

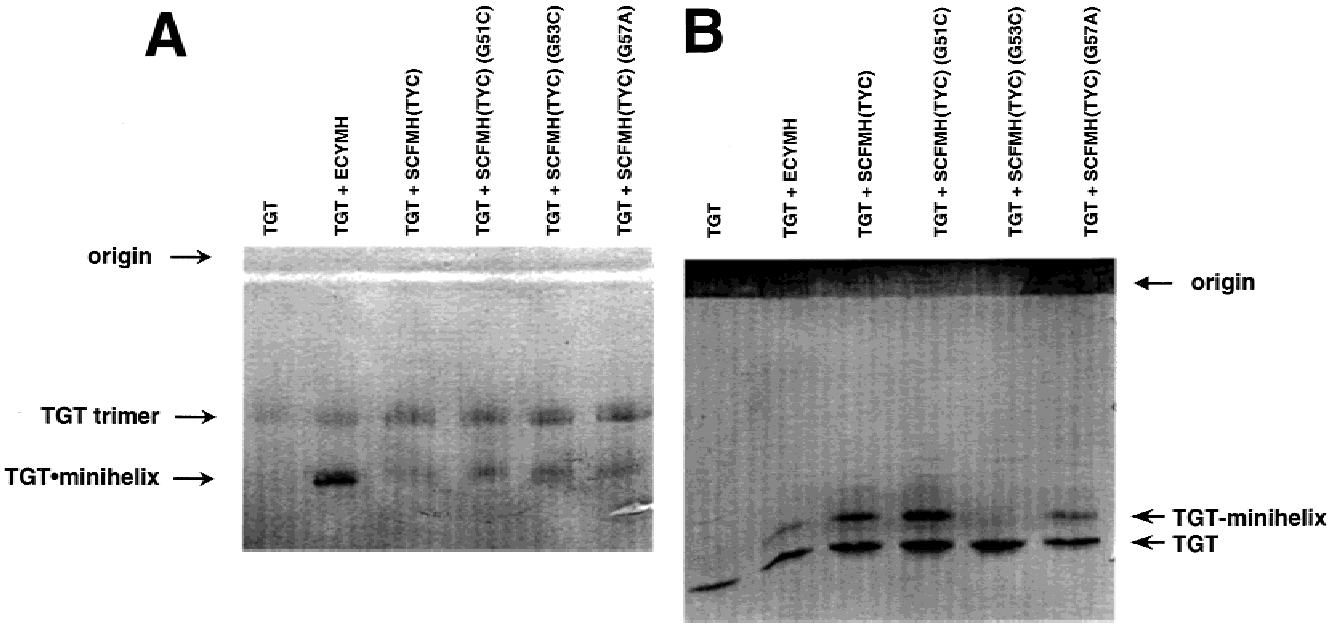

FIGURE 6. Native and denaturing PAGE of TGT and TGT•minihelical RNA complexes. Gels were run as in Figures 2–4 with the exception that the gel in **B** was silver stained. **A**: Native-PAGE; **B**: Denaturing-PAGE.

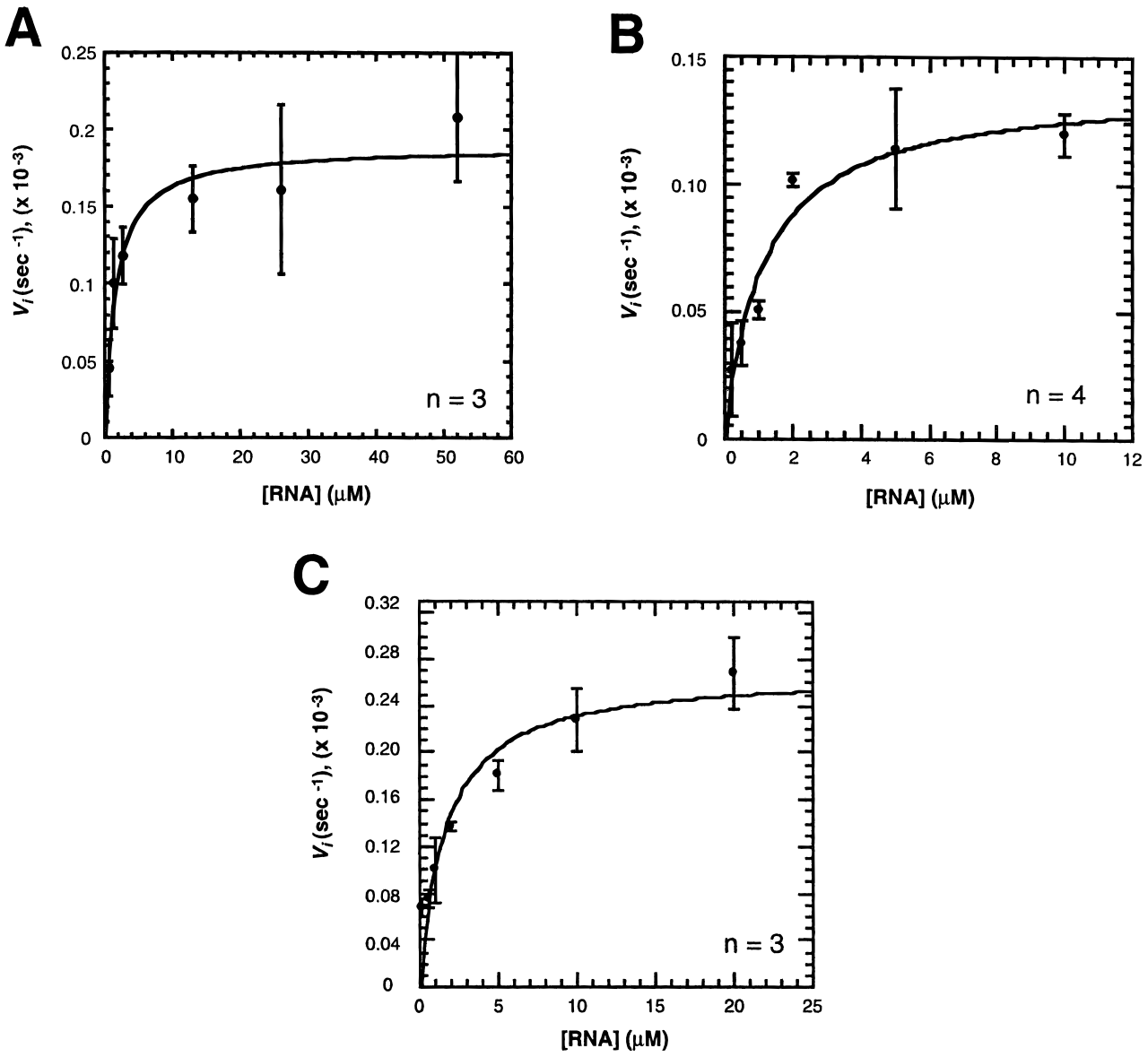

FIGURE 7. Michaelis-Menten analyses of the minihelical RNAs. **A**: SCFMH(TΨC); **B**: SCFMH(TΨC) (G51C); **C**: SCFMH(TΨC) (G57A). The averages of data points obtained from two, three, or four independent determinations are plotted. The curves represent fits of the data calculated by nonlinear regression. Error bars are generated from the standard deviation within each point.

change reaction, a higher affinity between a substrate tRNA and the enzyme also means a slower product release step. As a result, the turnover of the enzyme would be reduced, and thus a lower k_{cat} would be observed if the product dissociation step is rate limiting. Although this is not known for TGT, rate-limiting product release has been demonstrated for ribonuclease P (Beebe & Fierke, 1994), an enzyme that also involves tRNA as a product.

An alternative explanation is provided by the discovery of a second TGT recognition site within the tRNA^{Phe} TΨC arm discussed below. This second site involves a less productive interaction with TGT (that is k_{cat} is lower, see below). If this second site is active within the chimeric tRNA (SCF/D) then the kinetic parameters that we have determined would represent a combination of kinetic parameters for both sites and could then yield apparent K_m and k_{cat} lower than those for queuine-cognate tRNAs. Support for this second mode of interaction with TGT comes from the observation of a second, faint TGT•SCF/D complex band in the native-PAGE band-shift experiment. The second band (migrating to a slightly lower M_r) could be due to a conformationally different complex (and hence a slightly different migration under native-PAGE conditions) between TGT and a second site in SCF/D. In any event, these results suggest that there are no negative TGT recognition elements in the body of tRNA^{Phe}, a queuine

TABLE 3. Kinetic parameters for minihelical RNAs.

Analog	K_m[a,b] (μM)	k_{cat}[a,b] (10^{-3}•s^{-1})	k_{cat}/K_m (10^{-3}•s^{-1}•μM^{-1})	relative k_{cat}/K_m[c]
ECYMH	4.68 (1.61)	2.32 (0.26)	0.50 (0.23)	0.37
SCFMH(TΨC)	1.57 (0.49)	0.19 (0.01)	0.12 (0.05)	0.088
SCFMH(TΨC) G51C	1.41 (0.42)	0.14 (0.01)	0.10 (0.04)	0.074
SCFMH(TΨC) G53C	NA[d]	NA	NA	NA
SCFMH(TΨC) G57A	1.64 (0.40)	0.27 (0.02)	0.16 (0.05)	0.12

[a] Standard errors are shown in parentheses.
[b] Kinetic parameters are determined from the average of two (ECYMH), three [SCFMH(TΨC) and SCFMH(TΨC) (G57A)], or four [SCFMH(TΨC) (G51C)] replicate determinations of initial velocity data.
[c] The relative k_{cat}/K_m was calculated relative to that for ECY (Table 1).
[d] No detectable activity at concentrations up to 25 μM.

noncognate tRNA, although this is somewhat confounded by the second recognition site.

Control experiments with in vitro-transcribed tRNA^{Phe} [SCF(in vitro)] have surprisingly shown that this unmodified, queuine-noncognate tRNA is recognized by TGT. In vitro assays with TGT show that even though the kinetic parameters for SCF/D are significantly lower than those for queuine-cognate tRNA, this tRNA is a true substrate, exhibiting Michaelis-Menten kinetics. The reverse chimeric tRNA SCD/F exhibits very low activity with TGT. Control experiments with a totally noncognate tRNA, tRNA^{Ala} from the archaeon *A. fulgidus*, suggest that TGT is capable of catalyzing guanine exchange into noncognate tRNAs in a nonspecific manner. This occurs under fairly extreme conditions of high enzyme concentration (~0.25 μM) and long incubation times (up to 4 h) with about 1% of the normal TGT activity. (It should be noted that under optimal conditions, TGT is a relatively slow enzyme ($k_{cat} \sim 5 \times 10^{-3}$ s^{-1}). Practically, this makes it very difficult to accurately determine activities below 1% of that for wild-type enzyme and substrates under Michaelis-Menten conditions, although single-turnover kinetic studies should be possible.) We conclude that the activity observed for SCD/F is due to a nonspecific incorporation of guanine into tRNA by TGT, and that this tRNA is not a true substrate for TGT.

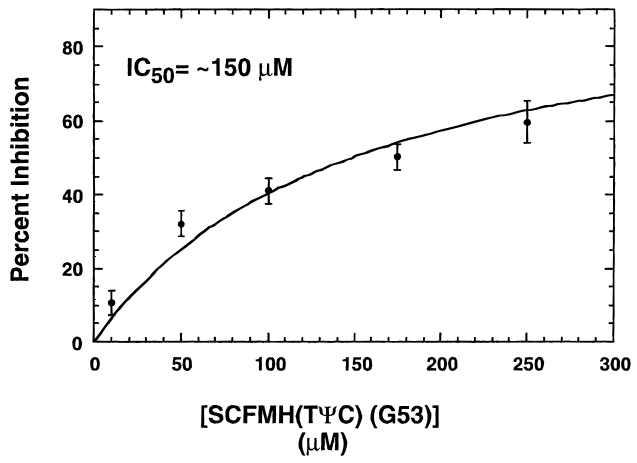

FIGURE 8. Inhibition constant plot for the inhibition of TGT by SCFMH(TΨC) (G53C). The averages of data points obtained from three independent determinations are plotted. The curve represents a fit of the data calculated by nonlinear regression. Error bars are generated from the standard deviation within each point.

These results indicate that a TGT recognition site must exist in the body of tRNA^{Phe} that is absent in the body of tRNA^{Asp}. Previous studies (Nakanishi et al., 1994; Curnow & Garcia, 1995) have elucidated a minimal TGT recognition motif that consists of a U-G-U sequence in a loop at the end of a double helical stem. Manual inspection of the tRNA^{Phe} and tRNA^{Asp} sequences reveal that a sequence (U-G-U-G-U) exists in the stem-loop junction of the TΨC arm of tRNA^{Phe}. This sequence does not exist in the tRNA^{Asp}. It is reasonable to speculate that TGT retains its specificity for a U-G-U sequence, but within the tRNA^{Phe}, it has recognized that sequence in a different part of the tRNA molecule. To test this hypothesis, we chemically synthesized and characterized an oligoribonucleotide (SCFMH(TΨC) corresponding to the TΨC arm of tRNA^{Phe} [with a four-base extension of the helix into the acceptor arm to impart stability of the helix at our assay temperature of 37 °C (Curnow & Garcia, 1995)]. This minihelical RNA is indeed a substrate for TGT, exhibiting Michaelis-Menten kinetics. This demonstrates that TGT can recognize a site within the TΨC arm of tRNA^{Phe} and strongly suggests that this represents the site of TGT activity within tRNA^{Phe}. Three mutant SCFMH (TΨC) minihelical RNAs were designed, synthesized, and characterized to determine the precise position of TGT activity within SCFMH(TΨC). These mutants involved the inversion of base pairs G51:C63 (G51C mutant), G53:C51 (G53C mutant), and the mutation of G57 in the loop to A (G57A mutant). The results of TGT activity assays and native and denaturing-PAGE analyses are all consistent with G53 being the site of TGT

activity. The ability of the SCFMH(TΨC) G53C mutant to inhibit TGT-catalyzed incorporation of guanine into the minihelical substrate ECYMH indicates that this analog can still bind to TGT even in the absence of the guanine at position 53, albeit significantly more poorly than G53-containing analogs. This is consistent with the native-PAGE results that also indicate that the G53C mutant binds to TGT. It is tempting to speculate that any minihelical RNA will bind to TGT, however previous results (Curnow & Garcia, 1995; Nakanishi et al., 1994) along with the data reported here indicate that TGT requires the U-G-U sequence for recognition. Substitution of any of these three bases results in dramatically reduced recognition [note that the estimated K_i for the G53C mutant is ~100-fold higher than K_m for the wild-type SCFMH(TΨC)]. The most striking observation regarding the present report is that the U-G-U sequence can reside in a different structural context than was previously believed and can still be recognized by TGT. We are currently investigating the precise structural context requirements for recognition. Preliminary results suggest that the U-G-U sequence is recognized in only a few specific positions within a seven-base loop/helix structure (S.T. Nonekowski & G.A. Garcia, unpubl.).

To further investigate the recognition of this site by TGT, we obtained fully modified $tRNA^{Phe}$ [SCF(in vivo)] from yeast. In contrast to the in vitro-transcribed tRNAs that we have studied, this tRNA contains the normal modified nucleosides found in yeast tRNAs. We should note that yeast tRNAs do not contain queuine, nor does yeast express TGT; however the yeast tRNAs that correspond to the queuine-cognate tRNAs (Tyr, Asp, Asn and His) are essentially equivalent to the *E. coli* tRNAs as substrates for the *E. coli* TGT (Kung & Garcia, 1998). Our results show that SCF(in vivo) does not bind appreciably to TGT nor is it a true substrate for TGT, although it does display nonspecific activity. The differential activity of SCF(in vitro) versus SCF(in vivo) is presumably due to the presence of modified nucleosides in SCF(in vivo). The TΨC arm of tRNA is positioned at the outer edge of the "elbow" region of tRNA. For TGT to recognize the TΨC arm of SCF, the bases of the U-G-U sequence must be sufficiently flexible to interact with the enzyme. This region contains a number of modified nucleosides that are involved in tertiary interactions that stabilize the three-dimensional structure of the tRNA molecule (Kim et al., 1974; Robertus et al., 1974). We propose that because of the presence of modified bases, SCF(in vivo) is too stable for TGT to gain access to the bases of the TΨC arm. This proposal is not without precedent. The phenylalanine-tRNA synthetase from yeast has been found to recognize a truncated (D-loop missing) $tRNA^{Phe}$ only when the modified base m^7G46 is missing (Renaud et al., 1979). Recently, Giegé and coworkers have suggested that this is because of a destabilization of the tRNA (in the absence of m^7G46) that "would provide greater structural flexibility to the fragment, which is supported by UV melting data (Renaud et al., 1979), and therefore better adaptability to the synthetase" (Wolfson et al., 1999). While we have shown that this fully-modified tRNA is not a true substrate for TGT, it is possible that such tRNAs (or other RNA species) may serve as substrates in vivo where TGT could recognize the tRNA at an intermediate stage of its maturation before the interfering modified bases have been introduced. Indeed, Grosjean and coworkers have characterized a pseudouridine synthase that only acts on precursor tRNAs containing an intron in the anticodon loop (Szweykowska-Kulinska et al., 1994).

The results presented here demonstrate that tRNA-guanine transglycosylase is capable of incorporating guanine into other sites in tRNAs and potentially other RNA species in addition to queuosine-cognate tRNAs. (It is very likely that this alternative RNA recognition will also hold for $preQ_1$ incorporation, the physiological reaction catalyzed by the *E. coli* TGT.) Santi and coworkers have found that the methyltransferase responsible for the methylation of U54 to rT54 in tRNA (RUMT) is capable of methylating a site in 16S rRNA in vitro (Gu et al., 1994). This site is similar in sequence and secondary structure to the TΨC arm of tRNA. Although this methylation was not found to occur in vivo, both Santi's results and the results presented here strongly suggest that at least these two tRNA modifying enzymes may introduce modified bases into other RNAs. Ofengand and coworkers have also identified a pseudouridine synthase that recognizes both tRNA (position 32) and ribosomal RNA (position 764 in 23S rRNA) in vitro and in vivo (Wrzesinski et al., 1995). These findings along with the present report suggest that we should look beyond the posttranscriptional modification of tRNA alone for potential physiological roles of modified bases and the enzymes that are involved in their biosyntheses.

MATERIALS AND METHODS

Reagents

Restriction enzymes were from New England Biolabs and Boehringer Mannheim. T4 polynucleotide kinase was from either Gibco BRL or New England Biolabs. The G, C, A, and U RNA phosphoramidite monomers were purchased from PerSeptive Biosystems. The yeast $tRNA^{Phe}$ (fully modified) was purchased from Sigma. 8-[^{14}C]-guanine (56 mCi/mmol) and 8-[^{3}H]-guanine (10 Ci/mmol) were from Moravek Biochemicals. T7 RNA polymerase and TGT were overexpressed and purified as described previously (Grodberg & Dunn, 1988; Garcia et al., 1993; Chong & Garcia, 1994). Oligodeoxynucleotide syntheses and DNA sequencing were performed at the University of Michigan, Biomedical Research Resources Core Facility.

Construction of chimeric tRNA in vitro transcription clones

The chimeric yeast $tRNA^{Phe\text{-}Asp}$ gene (SCF/D) possesses the anticodon loop of the $tRNA^{Asp}$ and the body of the $tRNA^{Phe}$, whereas the reverse chimera (SCD/F) contains the anticodon loop of the $tRNA^{Phe}$ and the body of the $tRNA^{Asp}$. The in vitro-transcription clones for these chimeric tRNAs were each constructed from two PCR primers (FOR1 and REV1 or FOR1 and REV2) and an oligodeoxynucleotide (SCF-D or SCD-F) containing a T7 promoter and the chimeric tRNA gene (SCF-D or SCD-F) as previously described (Kung & Garcia, 1998) with the following modifications. For SCF-D, the PCR reactions were annealed at 61 °C, the restriction enzyme *Afl*III was used to cut the PCR products. pTZ18U was blunt-ended with *Sma*I followed by restriction by *Afl*III and T4 polynucleotide kinase phosphorylation prior to subcloning and ligation. For SCD-F, the PCR reactions were annealed at 45 °C, the restriction enzymes *Eco*RI and *Bam*HI were used to cut the PCR products and the vector pTZ18U. In both cases, the restriction fragments were isolated, ligated, and transformed into *E. coli* TG2 cells as previously described (Kung & Garcia, 1998). Plasmid preparations (QIAprep Spin Column, QIAgene) from randomly picked colonies were further screened using restriction enzyme digestions [*Eco*0109I for pSCF/D and *Ear*I for pSCD/F)]. The sequences of the selected plasmids (named pSCF/D and pSCD/F) were confirmed by dideoxy sequencing using pTZSEQ (Table 4) as the primer (performed at the University of Michigan, Biomedical Research Resources Core Facility).

Preparation and purification of the full-length tRNA transcripts

The unmodified chimeric tRNAs (SCF/D and SCD/F), the unmodified *Saccharomyces cerevisiae* $tRNA^{Phe}$ [SCF(in vitro)], and the queuine-noncognate $tRNA^{Ala}$ (AFA) were generated via T7 RNA polymerase catalyzed in vitro transcription using the linearized plasmids (pSCF/D, pSCD/F, p67FY0, and pAFA, respectively) as the templates for run-off transcription and purified by anion exchange chromatography as previously described (Kung & Garcia, 1998). The $tRNA^{Phe}$ in vitro transcription clone, p67FY0 (Sampson & Uhlenbeck, 1988), was a gift from Dr. O.C. Uhlenbeck (Department of Chemistry and Biochemistry, University of Colorado, Boulder). The in vitro transcription clone for the totally nonqueuosine-cognate $tRNA^{Ala}$ from *A. fulgidus* (pAFA) was provided by Jeffrey Kittendorf (unpublished work from this laboratory). Concentrations of tRNAs were determined spectrophotometrically using the extinction coefficients at 260 nm calculated from the base composition of each transcript and corrected for the hypochromicity effect (HCF = 1.3–1.4). Approximately 0.5 mg of each tRNA was obtained per milliliter of transcription reaction. Native PAGE (data not shown) indicates that each of the tRNAs has been purified to homogeneity.

Chemical synthesis of minihelical RNAs

Minihelical RNAs were synthesized by automated chemical synthesis performed on an Expedite nucleic acid synthesis system using the manufacturer's protocols and reagents (model 8909, PerSeptive Biosystems). The synthetic oligonucleotides were cleaved from the CPG supports by treatment with 1.5 mL of ethanolic ammonium hydroxide [3:1 (v/v) 30% NH_4OH:ethanol) and then base deprotected by heating at 55 °C for 8 to 16 h. The crude RNA oligos were then 2′-hydroxyl deprotected by treatment with 600 μL of 1 M tetrabutylammonium fluoride (TBAF) in tetrahydrofuran (THF) (Aldrich) at room temperature for 24 h. The reactions were quenched by adding 600 μL of 1 M triethylammonium acetate (TEAA). The RNAs were then desalted by passing through an Oligonucleotide Purification Cartridge (OPC cartridge, Perkin Elmer Applied Biosystems Division) following protocols provided by the manufacturer. Oligos were eluted from the OPC cartridges with 1 mL of 50% acetonitrile and then dried by vacuum centrifugation at room temperature. The pellets

TABLE 4. Sequences of synthetic oligodeoxynucleotides.

Oligo	Sequence (5′–3′)[a]	Length (number of bases)	*Tm*[b] (°C)
SCF-D	CCCACATGTAATACGACTCACTATAGCGGATTTA GCTCAGTTGGGAGAGCGCCAGA**TTGTCGC**TCTG GAGGTCCTGTGTTCGATCCACAGAATTCGCACCA	101	n/a[c]
FOR1	CCCACATGTAATACGACTCAC	21	62
REV1	TGGTGCGAATTCTGTGGATCG	21	64
SCD-F	CCCACATGTAATACGACTCACTATAGCCGTGATA GTTTAATGTGCAGAATGGGCGC**CTGAAGA**GTGC CAGATCGGGGTTCAATTCCCCGTCGCGGCGCCA	100	n/a[c]
REV2	CTATAGGGAATTCCTAGACCTGGCGCCGCG	30	96 (38)
pTZSEQ	ACGCCAGGGTTTTCCCA	17	54

[a] T7 promoter region is underlined, the anticodon loop regions are in bold, and the outlined bases in REV2 overlap with SCD-F.

[b] The determinations of *Tm*, based on the following formula: *Tm* (°C) = 4(number of G + number of C) + 2(number of A + number of T), are for PCR primers only.

[c] n/a: not applicable.

were resuspended in 100 μL ddH_2O, ethanol precipitated, and then resuspended in 300–800 μL of 10 mM HEPES, pH 7.4, and 0.5 mM $MgCl_2$. Concentrations of the minihelical RNAs were determined spectrophotometrically using the extinction coefficients at 260 nm calculated from the base composition of each RNA. The extinction coefficients were later corrected for the hypochromicity effect (HCF = 1.3).

Polyacrylamide gel electrophoresis (PAGE)

Native and denaturing polyacrylamide gel electrophoresis was performed on a PhastSystem (Pharmacia) as previously described (Curnow & Garcia, 1994, 1995). Purity and homogeneity of the tRNAs were assessed via native polyacrylamide gel electrophoresis (data not shown).

To assess noncovalent binding of the tRNAs to TGT, native PAGE band-shift assays were performed as follows: 3 μM TGT were preincubated with 42 μM tRNA at 37 °C for 30 min in a reaction mixture containing 10 mM HEPES, pH 7.4, 10 mM $MgCl_2$, and 1 mM EDTA. The reaction mixtures were then analyzed by native PAGE using 8–25% gradient polyacrylamide gels (Pharmacia). The gels were first stained by ethidium bromide for tRNA visualization and then by Coomassie blue for protein visualization (Fig. 2).

The ability of the tRNAs to form a stable complex with TGT [presumed to be a covalent intermediate in the TGT reaction (Romier et al., 1996b)] was probed via denaturing PAGE band-shift assays as follows: 3 μM TGT were preincubated with 42 μM tRNA at 37 °C for 30 min in a reaction mixture containing 10 mM HEPES, pH 7.4, 10 mM $MgCl_2$, and 1 mM EDTA. Ten microliters of SDS (5%), β-mercaptoethanol (10%), and bromophenol blue (0.02%) were added to each sample and they were incubated for an additional 1 h at 37 °C. Approximately 4 μL were loaded in each lane. The gels were stained with Coomassie blue to visualize the bands containing protein (Figs. 4 and 7A). The gel in Figure 6B was silver stained following the vendor's (Pharmacia) protocols.

Kinetic analyses

A guanine incorporation assay (TGT assay) was used to obtain the steady-state kinetic parameters (Tables 1 and 3) as previously described (Curnow & Garcia, 1995; Kung & Garcia, 1998). The concentrations of tRNAs ranged from 0.14 to 20 μM for SCF(in vitro), from 0.0325 to 5.2 μM for SCF/D, and from 0.13 to 52 μM for ECYMH and SCFMH(TΨC) and its analogs. Initial velocities (v_is), obtained from linear regression of guanine incorporation versus time for various concentrations of tRNA substrates, were plotted against substrate (tRNA) concentrations (Figs. 5 and 8). Assays were conducted in replicate (n = 2, 3, or 4; see Figs. 5 and 8). The average of data points (v_i) and the error bars generated from the standard deviation within each point were plotted. A control experiment, in which the initial velocity for *E. coli* $tRNA^{Tyr}$ at saturating concentration (26 μM) was obtained, was performed for each assay to normalize the specific activity of TGT from assay to assay. V_{max} and K_m were obtained by nonlinear regression of the hyperbolic plots. k_{cat} was obtained by dividing the V_{max} value by the TGT concentration (i.e., 250 nM in all TGT assays) and the aliquot volume (70 or 100 μL depending upon the level of activity).

The activities for SCF(in vivo) and SCD/F were too low to accurately determine kinetic parameters. For comparison, the relative initial velocities of guanine incorporation were determined for a series of tRNAs (Table 2). The tRNA concentrations were all at 25 μM with 250 nM TGT, 20 μM 8-[^{14}C]-guanine in 100 mM HEPES, pH 7.4, 20 mM $MgCl_2$, and 10 mM DTT. Aliquots were taken at various points over time ranges from 12 min (ECY) to 4 h (SCD/F). The tRNA-labeled AFA is an in vitro transcript of the $tRNA^{Ala}$ from *A. fulgidus*. It contains no UGU sequences. This was used as a totally noncognate tRNA controlling for nonspecific TGT activity.

Inhibition of TGT by SCFMH(TΨC) (G53C)

The ability of SCFMH(TΨC) (G53C) to inhibit the TGT reaction was assessed by monitoring the initial rate of TGT-catalyzed guanine incorporation into ECYMH (at 0.5 μM) under the standard condition described above at concentrations of SCFMH(TΨC) (G53C) varying from 10 to 250 μM. The IC_{50} for SCFMH(TΨC) (G53C) was calculated from a nonlinear regression of the percent inhibition data (averages of three independent experiments) to the following equation:

$$\% \text{ inhibition} = 100 \times [\text{inhibitor}]/(IC_{50} + [\text{inhibitor}]).$$

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REFERENCES

Beebe JA, Fierke CA. 1994. A kinetic mechanism for cleavage of precursor tRNA(Asp) catalyzed by the RNA component of *Bacillus subtilis* ribonuclease P. *Biochemistry 33*:10294–10304.

Behlen LS, Sampson JR, DiRenzo AB, Uhlenbeck OC. 1990. Lead-catalyzed cleavage of yeast $tRNA^{Phe}$ mutants. *Biochemistry 29*: 2515–2523.

Chong S, Garcia GA. 1994. A versatile and general prokaryotic expression vector, pLACT7. *BioTechniques 17*:686–691.

Curnow AW, Garcia GA. 1994. tRNA-guanine transglycosylase from *Escherichia coli*: Recognition of dimeric, unmodified $tRNA^{Tyr}$. *Biochimie 76*:1183–1191.

Curnow AW, Garcia GA. 1995. tRNA-guanine transglycosylase from *Escherichia coli*: Minimal tRNA structure and sequence requirements for recognition. *J Biol Chem 270*:17264–17267.

Curnow AW, Kung FL, Koch KA, Garcia GA. 1993. tRNA-guanine transglycosylase from *Escherichia coli*: Gross tRNA structural requirements for recognition. *Biochemistry 32*:5239–5246.

Dumas P, Ebel JP, Giegé, Moras D, Thierry JC, Westhof E. 1985. Crystal structure of yeast $tRNA^{Asp}$: Atomic coordinates. *Biochimie 67*:597–606.

Garcia GA, Goodenough-Lashua DM. 1998a. Appendix III: General properties of RNA-modifying and -editing enzymes. In: Grosjean H, Benne R, ed. *Modification and editing of RNA.* Washington, DC: ASM Press. pp 555–560.

Garcia GA, Goodenough-Lashua DM. 1998b. Mechanisms of RNA-modifying and -editing enzymes. In: Grosjean H, Benne R, ed. *Modification and editing of RNA*. Washington, DC: ASM Press. pp 135–168.

Garcia GA, Koch KA, Chong S. 1993. tRNA-guanine transglycosylase from *Escherichia coli*: Overexpression, purification, and quaternary structure. *J Mol Biol 231*:489–497.

Grädler U, Ficner R, Garcia GA, Stubbs MT, Klebe G, Reuter K. 1999. Mutagenesis and crystallographic studies of *Zymomonas mobilis* tRNA-guanine transglycosylase to elucidate the role of serine 103 for enzymatic activity. *FEBS Lett 454*:142–146.

Grodberg J, Dunn JJ. 1988. *ompT* encodes the *Escherichia coli* outer membrane protease that cleaves T7 RNA polymerase during purification. *J Bacteriol 170*:1245–1253.

Gu XG, Ofengand J, Santi DV. 1994. In vitro methylation of *Escherichia coli* 16S rRNA by tRNA (m^5U54)-methyltransferase. *Biochemistry 33*:2255–2261.

Hoops GC, Townsend LB, Garcia GA. 1995. tRNA-guanine transglycosylase from *Escherichia coli*: Structure-activity studies investigating the role of the aminomethyl substituent of the heterocyclic substrate $preQ_1$. *Biochemistry 34*:15381–15387.

Kasai H, Nakanishi K, Macfarlane RD, Torgerson DF, Ohashi Z, McCloskey JA, Gross HJ, Nishimura S. 1976. The structure of Q nucleoside isolated from rabbit liver transfer ribonucleic acid. *J Am Chem Soc 98*:5044–5046.

Kim SH, Sussman JL, Suddath FL, Quigley GJ, McPherson A, Wang AH, Seeman NC, Rich A. 1974. The general structure of transfer RNA molecules. *Proc Natl Acad Sci USA 71*:4970–4974.

Kung F-L, Garcia GA. 1998. tRNA-guanine transglycosylase from *Escherichia coli*: The effect of tRNA primary structure on recognition. *FEBS Lett 431*:427–432.

Limbach PA, Crain PF, McCloskey JA. 1994. Summary: The modified nucleosides of RNA. *Nucleic Acids Res 22*:2183–2196.

Muramatsu T, Nishikawa K, Nemoto F, Kuchino Y, Nishimura S, Miyazawa T, Yokoyama S. 1988a. Codon and amino-acid specificities of a transfer RNA are both converted by a single post-transcriptional modification. *Nature* (London) *336*:179–181.

Muramatsu T, Yokoyama S, Horie N, Matsuda A, Ueda T, Yamaizumi Z, Kuchino Y, Nishimura S, Miyazawa T. 1988b. A novel lysine-substituted nucleoside in the first position of the anticodon of minor isoleucine tRNA from *Escherichia coli*. *J Biol Chem 263*:9261–9267.

Nakanishi S, Ueda T, Hori H, Yamazaki N, Okada N, Watanabe K. 1994. A UGU sequence in the anticodon loop is a minimum requirement for recognition by *Escherichia coli* tRNA-guanine transglycosylase. *J Biol Chem 269*:32221–32225.

Renaud M, Ehrlich R, Bonnet J, Remy P. 1979. Lack of correlation between affinity of the tRNA for the aminoacyl-tRNA synthetase and aminoacylation capacity as studied with modified $tRNA^{Phe}$. *Eur J Biochem 100*:157–164.

Reuter K, Chong S, Ullrich F, Kersten H, Garcia GA. 1994. Serine-90 is required for enzymic activity by tRNA-guanine transglycosylase from *Escherichia coli*. *Biochemistry 33*:7041–7046.

Robertus JD, Ladner JE, Finch JT, Rhodes D, Brown RS, Clark BFC, Klug A. 1974. Structure of yeast phenylalanine tRNA at 3 Å resolution. *Nature* (London) *250*:546–551.

Romier C, Reuter K, Suck D, Ficner R. 1996a. Crystal structure of tRNA-guanine transglycosylase: RNA modification by base exchange. *EMBO J 15*:2850–2857.

Romier C, Reuter K, Suck D, Ficner R. 1996b. Mutagenesis and crystallographic studies of *Zymomonas mobilis* tRNA-guanine transglycosylase reveal aspartate 102 as the active site nucleophile. *Biochemistry 35*:15734–15739.

Sampson JR, DiRenzo AB, Behlen LS, Uhlenbeck OC. 1990. Role of the tertiary nucleotides in the interaction of yeast phenylalanine tRNA with its cognate synthetase. *Biochemistry 29*:2523–2532.

Sampson JR, Uhlenbeck OC. 1988. Biochemical and physical characterization of an unmodified yeast phenylalanine transfer RNA transcribed in vitro. *Proc Natl Acad Sci USA 85*:1033–1037.

Szweykowska-Kulinska Z, Senger B, Keith G, Fasiolo F, Grosjean H. 1994. Intron-dependent formation of pseudouridines in the anticodon of *Saccharomyces cerevisiae* minor tRNA(Ile). *EMBO J 13*:4636–4644.

Westhof E, Dumas P, Moras D. 1985. Crystallographic refinement of yeast aspartic acid transfer RNA. *J Mol Biol 184*:119–145.

Westhof E, Dumas P, Moras D. 1988. Restrained refinement of two crystalline forms of yeast aspartic acid and phenylalanine transfer RNA crystals. *Acta Crystallogr A 44*:112–123.

Wolfson AD, Khvorova AM, Sauter C, Florentz C, Giegé R. 1999. Mimics of yeast $tRNA^{Asp}$ and their recognition by aspartyl-tRNA synthetase. *Biochemistry 38*:11926–11932.

Wrzesinski J, Nurse K, Bakin A, Lane BG, Ofengand J. 1995. A dual-specificity pseudouridine synthase: An *Escherichia coli* synthase purified and cloned on the basis of its specificity for Ψ746 in 23S RNA is also specific for Ψ32 in $tRNA^{Phe}$. *RNA 1*:437–448.