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tRNA recognition by tRNA-guanine transglycosylase from *Escherichia coli*: The role of U33 in U-G-U sequence recognition

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ABSTRACT

In eubacteria, the biosynthesis of queuine, a modified base found in the wobble position (#34) of tRNAs coding for Tyr, His, Asp, and Asn, occurs via a multistep pathway. One of the key enzymes in this pathway, tRNA-guanine transglycosylase (TGT), exchanges the genetically encoded guanine at position 34 with a queuine precursor, $preQ_1$. Previous studies have identified a minimal positive RNA recognition motif for *Escherichia coli* TGT consisting of a stable minihelix that contains a U-G-U sequence starting at the second position of its seven base anticodon loop. Recently, we reported that TGT was capable of recognizing the U-G-U sequence outside of this limited structural context. To further characterize the ability of TGT to recognize the U-G-U sequence in alternate contexts, we constructed mutants of the previously characterized *E. coli* $tRNA^{Tyr}$ minihelix. The U-G-U sequence was shifted to various positions within the anticodon loop of these mutants. Characterization of these analogs demonstrates that in addition to the normal $U_{33}G_{34}U_{35}$ position, TGT can also recognize the $U_{34}G_{35}U_{36}$ analog (UGU^{+1}). The other analogs were not active. This indicates that the recognition of the U-G-U sequence is not strictly dependent upon its position relative to the stem. In *E. coli*, the full-length tRNA with a $U_{34}G_{35}U_{36}$ anticodon sequence is one of the isoacceptors that codes for threonine. We found that TGT is able to recognize $tRNA^{Thr(UGU)}$ but only in the absence of a uridine at position 33. U_{33}, an invariant base present in all tRNAs, has been shown to strongly influence the conformation of the anticodon loop of certain tRNAs. We find that mutation of this base confers on TGT the ability to recognize $U_{34}G_{35}U_{36}$, and suggests that loop conformation affects recognition. The fact that the other analogs were not active indicates that although TGT is capable of recognizing the U-G-U sequence in additional contexts, this recognition is not indiscriminate.

Keywords: antideterminants; queuine; substrate recognition; tRNA identity elements; tRNA modification

INTRODUCTION

Transfer RNA (tRNA), best known for its role in protein synthesis, is characterized by the presence of a variety of modified nucleosides. Indeed, of the over 90 modified nucleosides identified to date, a majority of them are found in tRNA (Limbach et al., 1994). The hypermodified base queuine [Q, (7-(4,5-*cis*-dihydroxy-1-cyclopenten-3-yl-aminomethyl)-7-deazaguanine)] is found in the wobble position (#34) of four tRNAs (Asp, Asn, His, and Tyr) with the anticodon sequence GUN (Harada & Nishimura, 1972; Tsang et al., 1983). In prokaroytes, the incorporation of queuine into tRNA occurs through a base exchange mechanism without cleavage of the phosphodiester backbone of the tRNA (Okada et al., 1979). $Guanine_{34}$ is removed and replaced with a queuine precursor, $preQ_1$ (7-aminomethyl-7-deazaguanine) by the enzyme tRNA-guanine transglycosylase (TGT E.C. 2.4.2.29; Okada & Nishimura, 1979; Okada et al., 1979; Noguchi et al., 1982). $PreQ_1$ is ultimately converted to queuine while attached to the tRNA (Reuter et al., 1991). In contrast to prokaryotes, eukaryotes cannot synthesize queuine and must obtain it from the diet (Kirtland et al., 1988). As a result, they produce Q-containing tRNA via a direct exchange of $guanine_{34}$ for queuine (Okada et al., 1976; Shindo-Okada et al., 1980). Although the incorporation of queuine occurs late in the tRNA maturation process (Nishikura & De Robertis, 1981), none of the other posttranscriptional modifications are required for recognition of the tRNA by TGT (Curnow et al., 1993).

Studies performed using truncated tRNAs have identified a minimal recognition motif that consists of a stable minihelix with a seven-base loop that contains a

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U_{33}-G_{34}-U_{35} sequence (Nakanishi et al., 1994; Curnow & Garcia, 1995). These studies also demonstrated that loop size was important for proper recognition owing to the fact that decreasing or increasing the loop size resulted in a loss of activity. The X-ray crystal structure of TGT from *Zymomonas mobilis*, which shows approximately 60% sequence identity to *Escherichia coli* TGT, has recently been solved (Romier et al., 1996a). Although the TGT•tRNA cocrystal structure has not yet been solved, a model using the coordinates of $tRNA^{Asp}$ obtained from the $tRNA^{Asp}$•aspartyl-tRNA synthetase complex (Ruff et al., 1991) was generated (Romier et al., 1996a). The model depicts TGT binding primarily to the anticodon arm of the tRNA, while the remainder of the tRNA is largely solvated. This is consistent with biochemical studies that demonstrate that TGT is capable of recognizing minihelical RNAs (Nakanishi et al., 1994; Curnow & Garcia, 1995).

Detailed kinetic analyses of the in vitro-transcribed cognate tRNAs from *E. coli* revealed that there are no additional elements that stand out as major recognition determinants (Kung & Garcia, 1998). These studies also included the transcripts for the corresponding tRNAs from *Saccharomyces cerevisiae*, in part, to determine if there was something intrinsic about yeast tRNA that could account for the lack of queuine in that organism (Walden et al., 1982). The yeast tRNAs displayed similar kinetics to the *E. coli* tRNAs as substrates for *E. coli* TGT, thereby ruling out this possibility, at least for unmodified transcripts (Kung & Garcia, 1998). The uniformity of the kinetic parameters for these eight tRNAs is consistent with a limited recognition motif consisting predominantly, if not exclusively, of the common U-G-U sequence.

The U-G-U sequence is also a major recognition determinant for the eukaryotic TGT. Experiments performed using tRNAs microinjected into *Xenopus laevis* oocytes have demonstrated that queuine can be incorporated into noncognate tRNAs, provided that the anticodon loop is mutated to contain U_{33}-G_{34}-U_{35} (Carbon et al., 1983). A similar mutation is also sufficient to confer activity in *E. coli*: a chimeric tRNA ($tRNA^{Phe/Asp}$) with the body of the noncognate yeast $tRNA^{Phe}$ and the anticodon loop sequence of the cognate yeast $tRNA^{Asp}$ was a substrate for *E. coli* TGT with kinetic parameters comparable to that of yeast $tRNA^{Asp}$ (Kung et al., 2000). Unexpectedly, during the course of these studies, it was discovered that TGT is capable of recognizing a U-G-U sequence outside the context of the U_{33}-G_{34}-U_{35} position of the anticodon loop.

Although there is much evidence that establishes the critical role that the U-G-U sequence plays in recognition by TGT, little work has been done to understand the precise structural context required for the recognition of this sequence. To further study the ability of TGT to recognize the U-G-U sequence in different contexts, we constructed mutants of the previously characterized *E. coli* $tRNA^{Tyr}$ minihelix (ECYMH; Curnow & Garcia, 1995). The position of the U-G-U sequence within the anticodon loop was shifted either forward toward the 3′ end or backward toward the 5′ end (see Fig. 1). Our results are most consistent with the conformation of the anticodon loop (mediated by U_{33}) influencing the ability of TGT to recognize the U-G-U sequence. We propose a dual function for U_{33} involving both direct and indirect recognition by TGT.

RESULTS

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

Previously, we have shown that under native polyacrylamide gel electrophoresis (PAGE) conditions, *E. coli* TGT migrates as a heterotrimer; however, in the presence of tRNA, it dissociates to form a monomeric TGT•tRNA complex that is observed as a shifted (to lower M_r) protein band on native PAGE (Curnow & Garcia, 1994; Reuter et al., 1994). This band shift phenomenon can be used to investigate the gross interactions between TGT and various tRNA and RNA minihelix analogs (Curnow & Garcia, 1994; Kung et al., 2000). To probe the effect of altering the context of the U-G-U sequence on TGT recognition, analogs of ECYMH were chemically synthesized in which the U-G-U sequence was shifted (both forward and backward) around the anticodon loop (Fig. 1). As shown in Figure 2A, UGU^{+1} exhibits a degree of band shift-

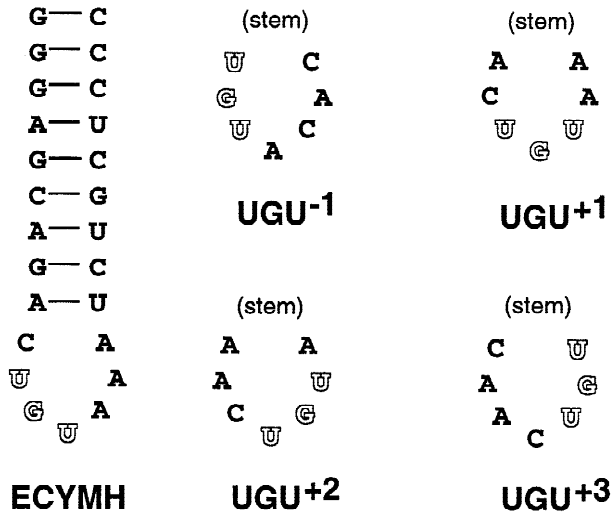

FIGURE 1. Secondary structures of the minihelix analog of *E. coli* $tRNA^{Tyr}$ (ECYMH) and the UGU-shifted analogs. The native minihelix, ECYMH, consists of the anticodon loop and stem of *E. coli* $tRNA^{Tyr}$ with a three-base extension to ensure stability at 37 °C (Curnow & Garcia, 1995). The location of the U-G-U sequence of this minihelix was shifted backward toward the 5′ end or forward toward the 3′ end. These shifted analogs are referred to as UGU^{-1}, UGU^{+1}, UGU^{+2}, and UGU^{+3}, respectively.

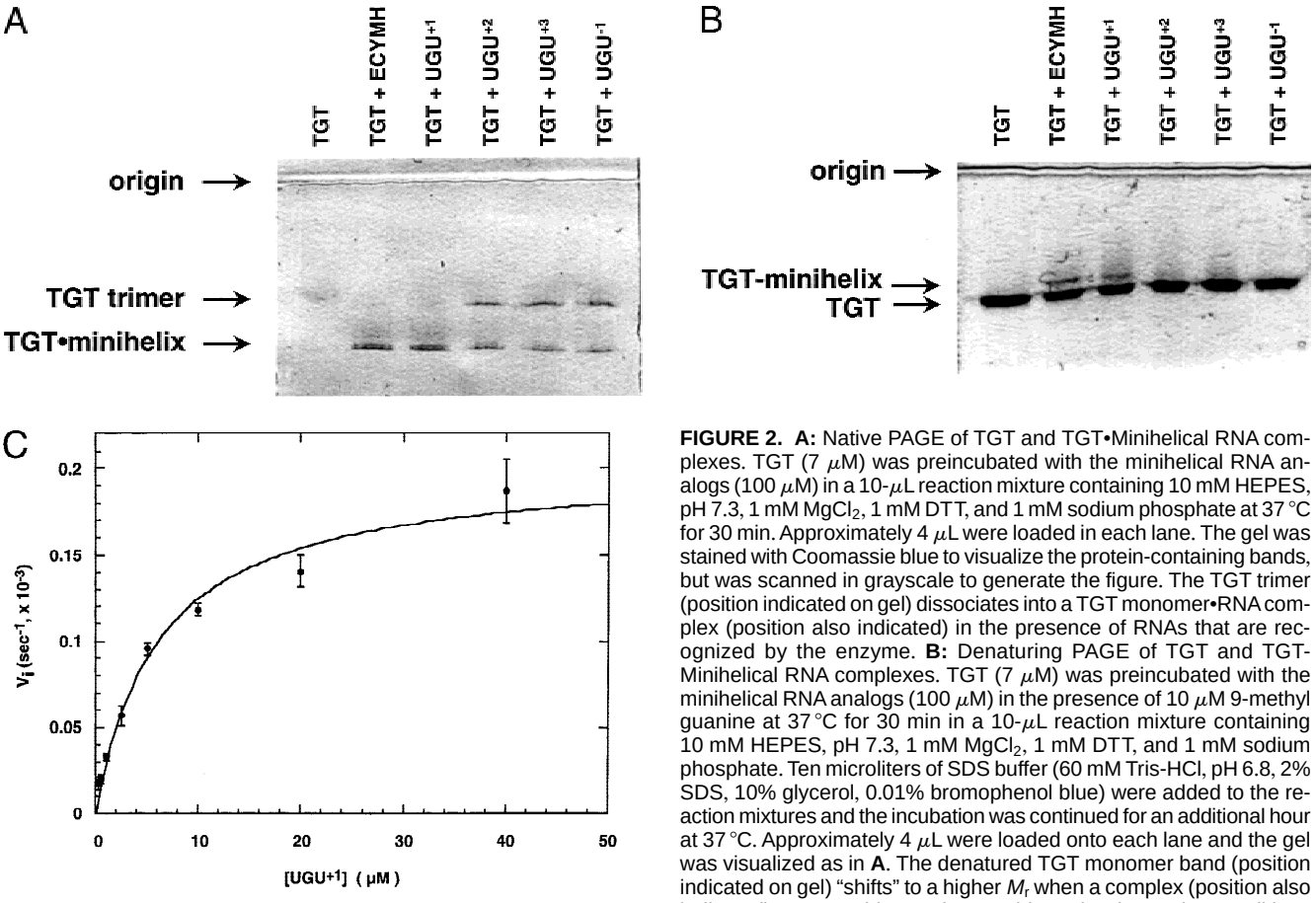

FIGURE 2. A: Native PAGE of TGT and TGT•Minihelical RNA complexes. TGT (7 μM) was preincubated with the minihelical RNA analogs (100 μM) in a 10-μL reaction mixture containing 10 mM HEPES, pH 7.3, 1 mM $MgCl_2$, 1 mM DTT, and 1 mM sodium phosphate at 37 °C for 30 min. Approximately 4 μL were loaded in each lane. The gel was stained with Coomassie blue to visualize the protein-containing bands, but was scanned in grayscale to generate the figure. The TGT trimer (position indicated on gel) dissociates into a TGT monomer•RNA complex (position also indicated) in the presence of RNAs that are recognized by the enzyme. **B:** Denaturing PAGE of TGT and TGT-Minihelical RNA complexes. TGT (7 μM) was preincubated with the minihelical RNA analogs (100 μM) in the presence of 10 μM 9-methyl guanine at 37 °C for 30 min in a 10-μL reaction mixture containing 10 mM HEPES, pH 7.3, 1 mM $MgCl_2$, 1 mM DTT, and 1 mM sodium phosphate. Ten microliters of SDS buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.01% bromophenol blue) were added to the reaction mixtures and the incubation was continued for an additional hour at 37 °C. Approximately 4 μL were loaded onto each lane and the gel was visualized as in **A**. The denatured TGT monomer band (position indicated on gel) "shifts" to a higher M_r when a complex (position also indicated), presumably covalent, stable to the denaturing conditions is formed with RNA. The observance of such a complex is directly correlated with TGT activity. **C:** Michaelis–Menten analysis of the minihelix analog UGU^{+1}: 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 were generated from the standard deviation within each point.

ing that is similar to the native ECYMH minihelix, whereas UGU^{+2}, UGU^{+3}, and UGU^{-1} show lower extents of band shifting.

Stable complex formation between minihelical RNA analogs and TGT

The formation of TGT-RNA complexes that are stable to mild denaturing conditions has been previously reported (Romier et al., 1996b; Kung et al., 2000). The ability of the UGU-shift analogs to form this stable complex was monitored via denaturing PAGE. Bands consistent with complex formation (~50 kDa) are clearly seen for ECYMH and UGU^{+1} (Fig. 2B), but are not observed for the other analogs.

Kinetic parameters of minihelical analogs with TGT

Kinetic results indicate that UGU^{+1} is a substrate for TGT with a k_{cat} that is only 10-fold lower than that for the ECYMH minihelix, and a K_M that remains essentially unchanged (Table 1). The plot of the initial velocities versus various RNA concentrations (Fig. 2C) demonstrates that UGU^{+1} follows Michaelis–Menten kinetics. UGU^{+2}, UGU^{+3}, and UGU^{-1} did not show any significant activity with TGT. When the guanine exchange assay was followed out to 4 h, a low level of activity was seen for UGU^{+2}, UGU^{+3}, and UGU^{-1}. However, even at these extended times, the activity was less than 2% of the activity of ECYMH and was assumed to result from nonspecific TGT activity, as noted previously (Kung et al., 2000).

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

Shifting the UGU sequence forward by one base places the UGU sequence in the anticodon position. In *E. coli*, this tRNA corresponds to one of the four isoacceptors that code for threonine (ECT4; Sprinzl et al., 1996). However, the first two bases in the anticodon loop of

TABLE 1. Kinetic parameters for UGU^{+} shift analogs.

Analog	K_M[a,b] (μM)	k_{cat}[a,b] ($10^{-3}\cdot s^{-1}$)	k_{cat}/K_M ($10^{-3}\cdot s^{-1}\cdot \mu M^{-1}$)	Relative k_{cat}/K_M[c]
ECY[d]	3.63 (0.44)	4.92 (0.19)	1.36 (0.17)	1
ECYMH[d]	4.68 (1.61)	2.32 (0.26)	0.50 (0.18)	0.37
ECT	nda[e]	nda	nda	nda
ECT($U_{33}C$)	3.93 (0.91)	0.24 (0.02)	0.061 (0.015)	0.045
ECT($C_{32}A/U_{33}C$)	1.51 (0.38)	0.42 (0.01)	0.27 (0.07)	0.20
UGU^{+1}	6.22 (1.19)	0.20 (0.01)	0.032 (0.006)	0.024
UGU^{+2}	nda[e]	nda	nda	nda
UGU^{+3}	nda[e]	nda	nda	nda
UGU^{-1}	nda[e]	nda	nda	nda

[a]Standard errors are shown in parentheses. Standard errors for k_{cat}/K_M were calculated as follows:

$$SE(k_{cat}/K_M) = k_{cat}/K_M \times \sqrt{[(SE\ k_{cat})/k_{cat}]^2 + [(SE\ K_M)/K_M]^2}.$$

[b]Kinetic parameters are determined from the average of two (ECYMH) or three (ECY, ECT($U_{33}C$), ECT($C_{32}A/U_{33}C$), and UGU^{+1}) replicate determinations of initial velocity.
[c]The relative k_{cat}/K_M was calculated relative to that for ECY.
[d]Kinetic parameters for ECY and ECYMH are from Kung et al. (2000).
[e]There was no detectable activity for ECT at concentrations up to 50 μM and for UGU^{+2}, UGU^{+3}, and UGU^{-1} at concentrations up to 25 μM with assay times out to 4 h. The limit of detection of the guanine exchange assay is approximately $1 \times 10^{-6}\ s^{-1}$.

this tRNA differ from that of the chemically synthesized UGU^{+1} ($C_{32}U_{33}$ versus $A_{32}C_{33}$; see Fig. 3). Therefore, in addition to wild-type ECT, two other full-length analogs were constructed in which one or both of these bases were changed to match UGU^{+1}. The ability of TGT to recognize ECT, ECT($U_{33}C$), and ECT($C_{32}A/U_{33}C$) was determined by the band-shift assay as described for the minihelix analogs. Figure 4A shows that wild-type ECT, ECT($U_{33}C$), and ECT($C_{32}A/U_{33}C$) are all able to elicit the characteristic band shift when incubated with TGT, albeit at lower extents of band shifting than ECY.

Stable complex formation between full-length RNA analogs and TGT

The full-length analogs were also evaluated for their ability to form stable complexes with TGT under mild denaturing conditions as discussed for the minihelix analogs (Fig. 4B). Bands that are consistent with stable complexes (~70 kDa) are observed for the normal substrate, *E. coli* $tRNA^{Tyr}$ (ECY), ECT($U_{33}C$), and ECT($C_{32}A/U_{33}C$). No band is detectable for the wild-type ECT.

Kinetic parameters of full-length analogs with TGT

Kinetic analyses reveal that both ECT($U_{33}C$) and ECT($C_{32}A/U_{33}C$) are substrates for TGT and follow Michaelis–Menten kinetics (Fig. 4C,D). Conversely, ECT showed no activity at concentrations up to 50 μM and assay times out to 4 h (data not shown). ECT($C_{32}A/U_{33}C$) exhibits a 10-fold decrease in k_{cat} with respect to ECY. Interestingly, this is the same decrease seen for the minihelix analog UGU^{+1} with respect to the minihelix ECYMH (see Table 1). The

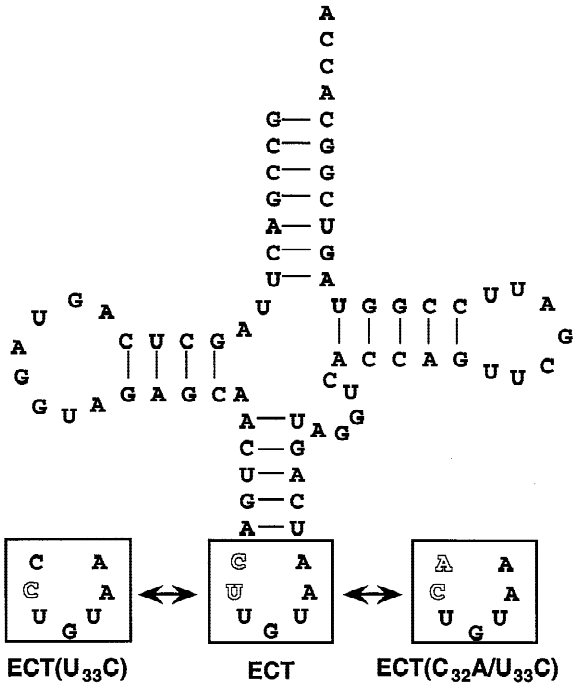

FIGURE 3. Secondary structures of *E. coli* $tRNA^{Thr}$ (wild-type ECT) and its anticodon loop analog ECT($C_{32}A/U_{33}C$). The sequence of *E. coli* $tRNA^{Thr}$ is from Sprinzl et al. (1996). One or both of the first two bases of the anticodon loop of ECT ($C_{32}U_{33}$) have been changed in ECT($U_{33}C$) and ECT($C_{32}A/U_{33}C$) to match the anticodon loop of UGU^{+1}.

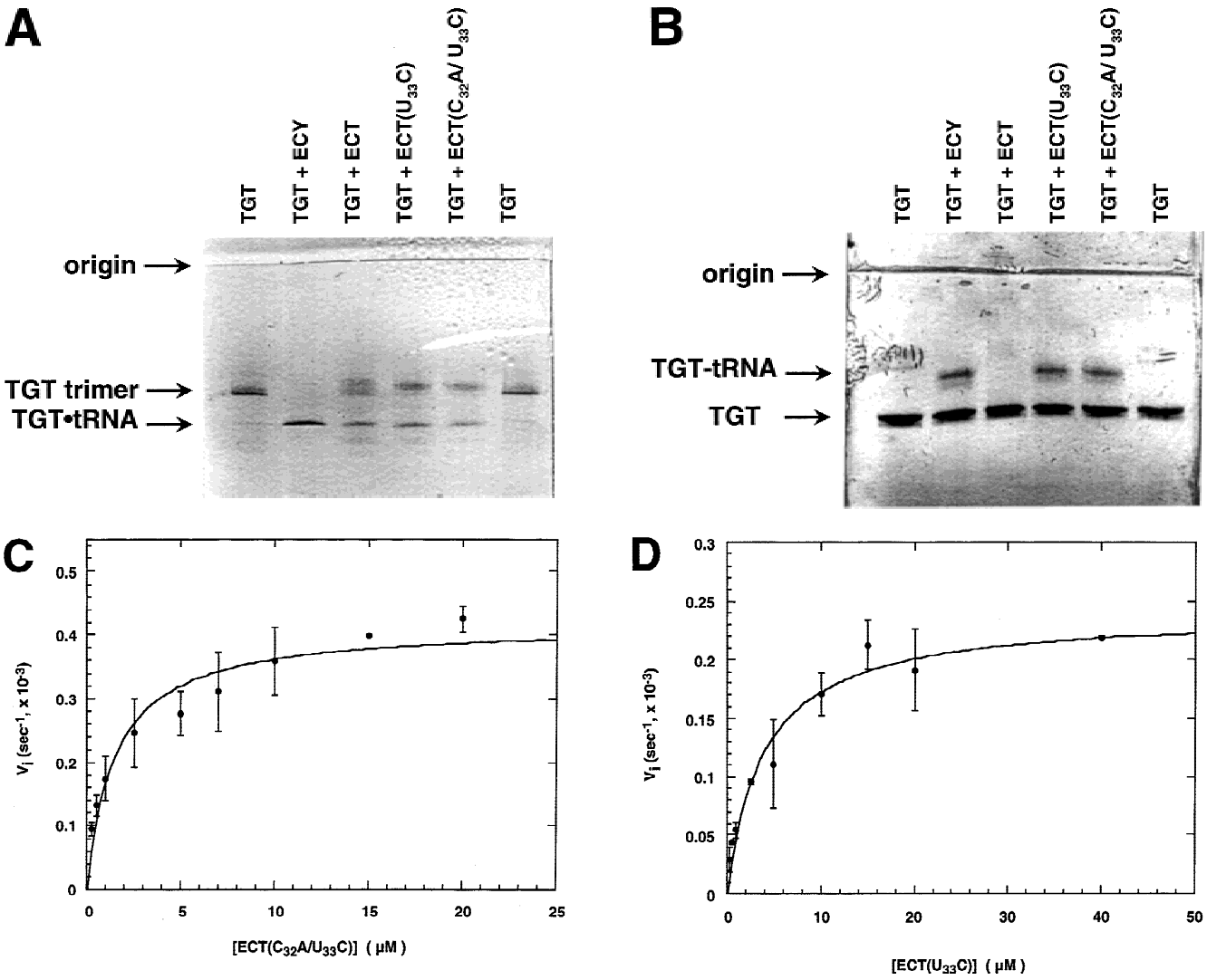

FIGURE 4. Native and denaturing PAGE of TGT•full-length tRNA complexes. The gels were run as described in Figure 2A,B with the exception that the TGT concentration was 3 μM and the tRNA concentrations were 45 μM. **A:** Native PAGE. **B:** Denaturing PAGE. **C,D:** Michaelis–Menten analyses of the full-length analogs ECT(U_{33}C) and ECT(C_{32}A/U_{33}C). The averages of data points obtained from three independent determinations are plotted. The curves represent a fit of the data calculated by nonlinear regression. Error bars were generated from the standard deviation within each point.

k_{cat} for ECT(U_{33}C) was about twofold less and the K_M was twofold higher than the respective kinetic parameters for the double mutant, ECT(C_{32}A/U_{33}C). A lower k_{cat} and a higher K_M is the same trend seen with the minihelix analog UGU^{+1}. Thus, the relative k_{cat}/K_M of ECT(U_{33}C) is similar to the minihelix analog UGU^{+1} and about 10-fold less than ECT(C_{32}A/U_{33}C).

DISCUSSION

Recent studies have revealed that the *E. coli* TGT is capable of recognizing the U-G-U sequence outside the context of the U_{33}-G_{34}-U_{35} position of the anticodon loop (Kung et al., 2000). Therefore, we constructed analogs in which the U-G-U sequence of the previously characterized *E. coli* tRNA^{Tyr} minihelix analog (ECYMH; Curnow & Garcia, 1995) was shifted to alternate locations in the anticodon loop. These analogs are delineated according to the number of bases shifted (1, 2, or 3) and the direction (toward the 3′ end (+) or towards the 5′ end (−)) of the shift (see Fig. 1). Native PAGE band-shift experiments reveal that UGU^{+1} and ECYMH bind to TGT with similar affinities (Fig. 2A). This suggests that the ability of TGT to recognize the U-G-U sequence when it has been shifted forward by one base is not dramatically impaired. In contrast, UGU^{+2}, UGU^{+3}, and UGU^{-1} show qualitatively less binding to TGT, suggesting that the U-G-U sequence in these analogs is in a less favorable location for binding.

The formation of a TGT-tRNA complex that is stable to mild denaturing conditions has been suggested to be indicative of a covalent mechanistic intermediate (Romier et al., 1996b). The authors postulate that this is a covalent intermediate formed by the nucleophilic attack of the enzyme upon the 1′ carbon of guanosine$_{34}$. Although it has not yet been established that this complex represents a true mechanistic intermediate, there is a direct correlation between the formation of this complex and enzymatic activity (Kung et al., 2000). Only UGU^{+1} was able to form an observable stable complex with TGT under mild denaturing conditions (Fig. 2B, lane 3). In vitro kinetic studies demonstrate that UGU^{+1} is a substrate for TGT with a k_{cat} that is 10-fold lower than for the ECYMH minihelix and a K_M that is only slightly higher (Table 1). UGU^{+2}, UGU^{+3}, and UGU^{-1} did not show any significant activity with TGT, which is consistent with their lack of ability to form a stable complex with TGT under mild denaturing conditions.

Examination of the sequence for UGU^{+1} reveals that the U-G-U sequence now resides in the anticodon position of the anticodon loop. In *E. coli*, the tRNA with the U-G-U anticodon sequence is one of the four isoacceptors that code for threonine (ECT4, hereafter referred to as ECT; Sprinzl et al., 1996). The modification pattern of ECT has not been determined. It is one of the minor isoacceptors for tRNA^{Thr}; therefore, it may not be present in sufficient quantities in vivo to allow RNA sequencing. As a result, it is not known whether the G$_{35}$ of ECT is modified in vivo. It is worth noting that tRNA$^{Thr(UGU)}$ is the major isoacceptor in *Bacillus subtilis*, yet queuine is not reported to be present in this tRNA. Instead, the U$_{34}$ is modified to 5-methoxyuridine (mo^{5}U; Hasegawa & Ishikura, 1978; Sprinzl et al., 1996). If TGT is unable to recognize a modified uridine in the U-G-U sequence, then it is possible that modification of U$_{34}$ serves to block in vivo recognition of tRNA$^{Thr(UGU)}$ by TGT in *B. subtilis*.

To determine if *E. coli* TGT could modify ECT in vitro, this tRNA was cloned from the reported gene sequence (Sprinzl et al., 1996). Owing to the fact that the first two bases in the anticodon loop of wild-type ECT differ from those of UGU^{+1} (Fig. 3), two other analogs, ECT(U$_{33}$C) and ECT(C$_{32}$A/U$_{33}$C), were also constructed and characterized. Although all three of these tRNAs were found to bind to TGT, only the two mutants were able to form complexes stable to SDS denaturation. Kinetic analyses reveal that both ECT(U$_{33}$C) and ECT(C$_{32}$A/U$_{33}$C) are substrates for TGT (Table 1). ECT(C$_{32}$A/U$_{33}$C) exhibits a 10-fold decrease in k_{cat} with respect to ECY that directly parallels the decrease seen for UGU^{+1} with respect to ECYMH. The kinetic parameters of ECT(U$_{33}$C) are similar to those for UGU^{+1}. In contrast, wild-type ECT showed no activity (Table 1), despite its significant binding at 45 μM (Fig. 4A, lane 3). Consistent with its ability to bind to TGT but not to serve as a substrate, 50 μM of ECT were able to inhibit 25% of the TGT-catalyzed incorporation of guanine into ECY (reaction mixtures contained 100 mM HEPES, pH 7.3, 20 mM $MgCl_2$, 5 mM DTT, 10 μM 8-[^{3}H]-guanine, 50 μM ECT, 1 μM ECY, and 50 nM enzyme; data not shown). This suggests that the loop conformation of wild-type ECT presents the U-G-U sequence in such a way that TGT is unable to perform catalysis. However, mutation of U$_{33}$ allows the loop to adopt a conformation that is catalytically active.

U$_{33}$ is an invariant base present in all tRNAs. In the X-ray crystal structure of yeast tRNA^{Phe}, U$_{33}$ plays a critical role in defining the conformation of the anticodon loop (Quigley & Rich, 1976). It is largely through rotation of the dihedral angle of this base that the polynucleotide chain of the tRNA is allowed to turn 180° within a short stretch of only 3 nt. This structural motif, also seen in the TΨC loop, was referred to as a U-turn (Quigley & Rich, 1976). The U-turn was verified by NMR studies of a RNA minihelix corresponding to the anticodon loop of yeast tRNA^{Phe} (Clore et al., 1984). Solution studies of analogs of initiator and elongator methionine tRNAs confirm the presence of the U-turn in the anticodon loops of other tRNAs (Schweisguth & Moore, 1997). U turns have also been documented both spectrally and by X-ray crystallography in 23S ribosomal RNA (Fountain et al., 1996; Huang et al., 1996), and in the active site of the hammerhead ribozyme (Pley et al., 1994; Scott et al., 1995). Based on these studies, a consensus sequence consisting of UNRN (where N is any base and R is any purine) has been described for U-turns (Moore, 1999). It is worth noting that although both yeast tRNA^{Phe} and *E. coli* tRNA^{Thr} (ECT) contain this consensus sequence (UNRN), none of the queuine cognate tRNAs do (UGUN). It is possible that the U-turn stabilizes the anticodon loop in a conformation that is unfavorable for recognition. The activity of ECT(U$_{33}$C) is consistent with this supposition. Presumably, mutation of C$_{32}$ as well as U$_{33}$ further increases the flexibility of the anticodon loop, as evidenced by the increased activity of the double mutant, ECT(C$_{32}$A/U$_{33}$C). Although the base at position 32 is not invariant, as is U$_{33}$, a pyrimidine at this position is highly conserved (98%; Auffinger & Westhof, 1999). This is thought to be due to the formation of a bifurcated hydrogen bond between a pyrimidine (Y) at position 32 and a purine (R) at position 38 of the anticodon loop (Quigley & Rich, 1976). Studies performed using amber suppressor tRNA have shown that purines at position 32 are strongly disfavored (Smith & Yarus, 1989). This noncanonical base pair (Y$_{32}$-R$_{38}$) is not isosteric if reversed, which might explain the predominance of pyrimidine 32 (Auffinger & Westhof, 1999). It is possible that the stabilizing effect of the Y$_{32}$-R$_{38}$ pair is responsible for the slightly lower k_{cat} and higher K_M of ECT(U$_{33}$C).

Our studies suggest that U_{33} has a dual function in TGT recognition. The first is to act as positive determinant for the cognate tRNAs, possibly through direct interaction in a pocket of the active site. Several amino acids that could directly interact with U_{33} were tentatively identified in the TGT•tRNA model (Romier et al., 1996a). This is consistent with mutagenic studies that demonstrated that replacement of U_{33} in the cognate tRNAs with any of the other bases resulted in a loss of activity (Nakanishi et al., 1994; Curnow & Garcia, 1995). A second function for U_{33} could be postulated in which U_{33} serves as a negative determinant for the noncognate tRNAs that contain a U-G-U sequence, such as ECT, via a conformational effect.

The molecular basis for U_{33} recognition by TGT is not presently clear. One possibility is that TGT directly recognizes the functional groups of U_{33}. This is the case for the discriminator base G_{73}, which acts as a positive determinant for aspartylation in both *E. coli* and yeast (Hasegawa et al., 1989; Putz et al., 1991). The crystal structure of yeast $tRNA^{Asp}$ complexed to its aspartyl-tRNA synthetase shows a tight interaction between G_{73} and the protein. Base-specific hydrogen bonds are present between G_{73} and residues 327–331 in the variable loop of motif 2 of the enzyme (Cavarelli et al., 1993). Biochemical data shows that replacement of G_{73} with U_{73} (which can maintain this contact with O-4) results in only a 36-fold decrease in aminoacylation compared to both A_{73} and C_{73}, which exhibit 160-fold and 200-fold decreases, respectively (Putz et al., 1991). A similar trend is seen in *E. coli* with 200- to 500-fold decreases in aminoacylation upon replacement of G_{73} with any of the other bases (Hasegawa et al., 1989). The crystal structure of *E. coli* $tRNA^{Asp}$ bound to its aspartyl-tRNA synthetase also shows base-specific contact of G_{73} (Eiler et al., 1999). Interestingly, one of the main differences between yeast $tRNA^{Asp}$ and *E. coli* $tRNA^{Asp}$ is the presence of Q_{34} in the latter. Hydrogen bonds were observed between the heterocyclic ring of Q_{34} and the synthetase, but no specific interactions were seen for the modification itself (Eiler et al., 1999). The authors postulate that Q_{34} could be functioning as an antideterminant for noncognate tRNA synthetases as evidenced by mutagenic studies (Martin et al., 1993).

There is evidence that in addition to serving as a positive recognition element for *E. coli* aspartyl-tRNA synthetase, G_{73} also serves as an antideterminant for *E. coli* methionyl-tRNA synthetase (Schulman, 1991). Changing the normal A_{73} in $tRNA^{fMet(CAU)}$ to a G_{73} results in an approximately 50% reduction in aminoacylation, whereas changing A_{73} to U_{73} has essentially no effect (Uemura et al., 1982). Meinnel et al. (1993) reported an even greater decrease in the efficiency of aminoacylation (by two orders of magnitude) when G_{73} analogs of methionyl-tRNAs were assayed in the presence of 150 mM KCl. Furthermore, the ability of methionyl-tRNA synthetase to charge a $tRNA^{Trp}$ derivative with a $tRNA^{fMet}$ anticodon sequence (CAU) is increased 10-fold when G_{73} is mutated (Schulman, 1991). Thus, the hypothesis that U_{33} functions as both a positive and negative RNA recognition element for TGT is not without precedent.

An alternative possibility is that TGT does not recognize U_{33} directly in noncognate tRNAs but instead is affected by the conformation of the anticodon loop. Evidence for this mechanism is provided by the fact that ECT(U_{33}C), which still has the pyrimidine at position 32, is less active than the double mutant, ECT(A_{32}C/U_{33}C). Structural recognition is also seen with *E. coli* valyl-tRNA synthetase, where a G_4-U_{69} base pair acts as a negative determinant because it disrupts the regular A-type helix geometry that is needed for recognition (Liu et al., 1997). Conversely, a G_3-U_{70} base pair inserted into tRNAs that are inactive for phenylalanylation is thought to add enough flexibility to the acceptor stem to allow productive interaction with yeast phenylalanyl-tRNA synthetase (Frugier et al., 1998). The structural role of U_{33} has also been postulated to account for the lack of aminoacylation seen with an *E. coli* $tRNA^{Val}$ mutant with its anticodon sequence shifted back one position ($U_{33}A_{34}C_{35}$) in contrast to the activity seen with the forward shifted mutant ($U_{35}A_{36}C_{37}$; Horowitz et al., 1999).

It is also quite possible that U_{33} achieves a dual function through a combination of both direct and indirect recognition of this base by TGT. There is evidence that the G•U wobble base pair found at positions 3 and 70 of the acceptor stem of *E. coli* $tRNA^{Ala}$ is directly (Musier-Forsyth et al., 1991; Musier-Forsyth & Schimmel, 1992) and indirectly (Gabriel et al., 1996; Mcclain et al., 1996) recognized. Although it is not clear at this point which mechanism governs the recognition of $tRNA^{Ala}$ by its synthetase, it is likely that both mechanisms play some role in determining the specificity of $tRNA^{Ala}$.

It appears that *E. coli* TGT specificity, as in many of the aminoacyl-tRNA synthetases, is the result of a combination of direct recognition of specific bases (the U-G-U sequence in cognate tRNAs) and structural elements (e.g., conformational differences) that influence its discrimination between cognate and noncognate tRNAs.

MATERIALS AND METHODS

Reagents

Reagents were purchased from Sigma, Aldrich, or Gibco BRL unless otherwise noted. Bactotryptone and yeast extract were from Difco Laboratories. Restriction enzymes were from New England Biolabs and Boehringer Mannheim. Nucleoside triphosphates were from Pharmacia. Inorganic pyrophosphatase was from Boehringer Mannheim. RNase Inhibitor was

from Gibco BRL. 8-[^{14}C]-Guanine (56 mCi/mmol) and 8-[^{3}H]-guanine (10 Ci/mmol) were from Moravek Biochemicals. Deoxyoligonucleotide syntheses and DNA sequencing were performed at the University of Michigan, Biomedical Research Resources Core Facility. RNA minihelices were chemically synthesized using G, C, A, and U RNA phosphoramidite monomers and CPG columns from Glen Research. All other reagents for automated RNA synthesis were from PerSeptive BioSystems. TGT and T7 RNA polymerase were overexpressed and purified as described previously (Grodberg & Dunn, 1988; Garcia et al., 1993; Chong & Garcia, 1994).

Chemical synthesis of minihelical RNAs

All synthetic minihelical RNA analogs were synthesized by automated chemical synthesis performed on an Expedite nucleic acid synthesis system (model 8909, PerSeptive Biosystems) using the manufacturer's protocols and reagents. The solid support was transferred from the column by removing the end-crimp and pouring it into a sterile 1.5 mL screw cap microfuge tube. The synthetic oligonucleotides were cleaved from the CPG support by treatment with 1.5 mL of 30% ethanolic ammonium hydroxide (3:1 (v/v) NH_4OH: ethanol) and then base deprotected by incubating at 55 °C for 8 to 16 h. The crude RNA oligos were 2′-hydroxyl deprotected by treatment with 600 μL of 1 M tetrabutylammonium fluoride (TBAF) in tetrahydrofuran (THF; Aldrich) at room temperature for 24–48 h. The reactions were quenched by the addition of 600 μL of 1 M triethylammonium acetate (TEAA). The THF was removed by vacuum centrifugation. The deprotected oligos were desalted using Oligonucleotide Purification Cartridges (OPC, Applied Biosystems) following the protocols provided by the manufacturer. Oligos were eluted from the OPCs with 1 mL of 50% acetonitrile and then dried by vacuum centrifugation at room temperature. The pellets were resuspended into 300 μL TE8 buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and ethanol precipitated. The resulting pellet was resuspended into 300–800 μL of HM 7.3 buffer (10 mM HEPES, pH 7.3, 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 and then corrected for hypochromicity using a correction factor (1.3–1.4) determined previously (Kung, 1998).

Construction of the full-length tRNA in vitro transcription clones

The full-length analogs, ECT, ECT(U_{33}C), and ECT(C_{32}A/U_{33}C), were generated as previously described (Kung & Garcia, 1998; Kung et al., 2000) with a few modifications. Each clone was constructed from two PCR primers, FOR1 and REV1 (Kung et al., 2000), and an oligonucleotide containing a T7 promoter upstream of either the *E. coli* $tRNA^{Thr}$ gene (wild-type ECT), the gene with the U_{33}-to-C_{33} mutation, ECT(U_{33}C), or the gene with the $C_{32}U_{33}$-to-$A_{32}C_{33}$ double mutation, ECT(A_{32}C/U_{33}C; Sprinzl et al., 1996). The PCR products (30 cycles of 94 °C, 1 min, 50 °C, 1 min, and 72 °C, 2 min) and the vector pTZ18U (Amersham Pharmacia) were doubly digested with *Eco*RI and *Bam*HI. The restrictions 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 screened using the restriction enzyme *Bst*EII, as these tRNA genes contain a unique site for this enzyme. The sequences of the selected plasmids, named pECT, pECT(U_{33}C), and pECT(C_{32}A/U_{33}C), were confirmed by dideoxy sequencing at the University of Michigan, Biomedical Research Resources Core Facility, using the primer pTZSEQ (Kung et al., 2000).

Preparation and purification of the full-length tRNA transcripts

The tRNAs were generated via T7 RNA polymerase catalyzed in vitro transcription using *Bst*NI linearized plasmids (pECT(U_{33}C), pECT(C_{32}A/U_{33}C), or pECT) as the templates for run-off transcription as previously described (Kung & Garcia, 1998). The transcription pellets were resuspended in 10 mL of either denaturing tRNA buffer-A for ECT (10 mM HEPES, pH 7.3, 1 mM EDTA, and 7 M urea) or native tRNA buffer A for ECT(U_{33}C) and ECT(C_{32}A/U_{33}C) (10 mM HEPES, pH 7.3). [Technical note: wild-type ECT transcripts were purified under denaturing conditions using 7 M urea. In an effort to improve the overall yield and streamline the purification process, a native purification protocol was used for ECT(U_{33}C) and ECT(C_{32}A/U_{33}C). Regardless of the purification protocol used, the tRNAs were purified to homogeneity as demonstrated by denaturing and native-PAGE (data not shown).] The transcripts were sterile filtered to remove any particulate matter and then applied to an anion exchange column (MonoQ HR10/10, Pharmacia) that had been pre-equilibrated with the appropriate buffer A. The elution profiles were developed as a gradient of 100% buffer A to 100% buffer B (Buffer A + 1 M NaCl) at 1 mL/min. ECT eluted at 50% denaturing tRNA-buffer B whereas ECT(U_{33}C) and ECT(C_{32}A/U_{33}C) eluted between 55–60% native tRNA-buffer B. Native fractions containing tRNA were pooled and concentrated by precipitating with ethanol. Urea was removed from denatured fractions by diluting the pooled fractions with two times the volume of ddH_2O and ethanol precipitating the samples overnight at −20 °C. This procedure was repeated at least three times to ensure that all the urea had been removed. The tRNA transcripts were resuspended to a concentration of less than 20 μM. Samples were heated to 70 °C for 30 min. $MgCl_2$ was added to a concentration of 1 mM and the samples were allowed to renature on ice for 2 h before ethanol precipitation overnight at −20 °C. Final pellets were resuspended in 5–10 mL of HM 7.3 buffer (10 mM HEPES, pH 7.3, 0.5 mM $MgCl_2$). Concentrations of the tRNAs were determined spectrophotometrically using corrected extinction coefficients as described above. Approximately 0.25 mg of ECT, 0.4 mg of ECT(U_{33}C), and 0.5 mg of ECT(C_{32}A/U_{33}C) were obtained per milliliter of transcription reaction.

Polyacrylamide gel electrophoresis

All native and denaturing PAGEs were performed on a Phast System (Pharmacia) as previously described (Curnow & Garcia, 1994, 1995). Purity and homogeneity of the RNAs was assessed via denaturing and native PAGE using Homo20 gels (Pharmacia; data not shown). Typical native band-shift assays were performed as follows: TGT (3–7 μM) was incubated with 15-fold excess RNA (45–100 μM) at 37 °C for

30 min in a 10-μL reaction mixture containing 10 mM HEPES, pH 7.3, 1 mM $MgCl_2$, 1 mM DTT, and 1 mM sodium phosphate. The reaction mixtures were then analyzed by native PAGE using 8–25% gradient polyacrylamide gels (Pharmacia). Approximately 4 μL was loaded onto each lane. The ability of the RNAs to form a stable complex with TGT was assayed via denaturing PAGE as follows: TGT (3–7) μM was incubated with 15-fold excess RNA (45–100 μM) in the presence of 10 μM 9-methyl guanine at 37 °C for 30 min in a 10 μL reaction mixture containing 10 mM HEPES, pH 7.3, 1 mM $MgCl_2$, 1 mM DTT, and 1 mM sodium phosphate. Ten microliters of SDS buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.01% bromophenol blue) were added to the reaction mixtures and the incubation was continued for an additional hour at 37 °C. Approximately 4 μL were loaded onto each lane. In both native and denaturing PAGE, the gels were stained with Coomassie blue to visualize the protein, although the gels were scanned in grayscale to generate the figures.

Kinetic analyses

A guanine incorporation assay (TGT assay) was used to obtain the steady-state kinetic parameters as previously described (Curnow & Garcia, 1995; Kung & Garcia, 1998; Kung et al., 2000). In a typical assay, the analogs were incubated at 37 °C in a reaction mixture composed of 100 mM HEPES, pH 7.3, 20 mM $MgCl_2$, 5 mM DTT, 250 nM enzyme, and 10 μM 8-[^{3}H]-guanine or 8-[^{14}C]-guanine. The RNA concentrations ranged from 0.25 to 40 μM for UGU^{+1}, from 0.25 to 20 μM for ECT($C_{32}A/U_{33}C$), and from 0.25 to 40 μM for ECT($U_{33}C$). Aliquots (70 μL) were taken at various times (usually 3, 6, 9, 12, 15, and 18 min) after the reaction had been initiated with enzyme and quenched by precipitation with either 2 mL of ethanol and 10 μL 3 M sodium acetate, pH 5.3 (in the case of the minihelices) or 2 mL of 5% trichloroacetic acid (for the full-length analogs). Incubation times up to 4 h and concentrations up to 25 μM for the minihelices and up to 50 μM for ECT were used to monitor these analogs for activity. The precipitated RNAs were collected on glass fiber filters (GF/C filter, Whatman), washed, dried, and quantitated by liquid scintillation (note: quench curves for both [^{3}H] and [^{14}C] were used to calculate DPMs, which were then converted to picomoles using the appropriate specific activities). Michaelis–Menten plots were generated by plotting the initial velocities ($v_i s$), obtained from linear regression of guanine incorporation versus time, versus substrate concentrations. 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 (250 nM) and the aliquot volume (70 μL). Assays were conducted in triplicate and the average of the data points (v_i) and the error bars generated from the standard deviation within each point were plotted. The initial velocities ($v_i s$) of either ECYMH or ECY at saturating concentrations were determined in all assays to normalize the specific activity of TGT from assay to assay.

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