The *Escherichia coli* tRNA-Guanine Transglycosylase Can Recognize and Modify DNA*

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The tRNA-guanine transglycosylase (TGT) catalyzes the exchange of queuine (or a precursor) for guanine 34 in tRNA. The minimal RNA recognition motif for TGT has been found to involve a UGU sequence in the anticodon loop of the queuine-cognate tRNAs. Recent studies have shown that the enzyme is capable of recognizing the UGU sequence in alternative contexts (Kung, F. L., Nonekowksi, S., and Garcia, G. A. (2000) RNA 6, 233–244) and have investigated the role of the first U of the UGU sequence in tRNA recognition by TGT (Nonekowksi, S. T., and Garcia, G. A. (2001) RNA 7, 1432–1441). The TGT reaction involves the breakage and re-formation of a glycosidic bond. To rule out a potential chemical mechanism involving the 2'-hydroxyl at position 34, we synthesized and evaluated an RNA minihelix with 2'-deoxyguanosine G at 34. The high level of activity exhibited by this analogue indicates that the 2'-hydroxyl of G34 is not required for catalysis. Furthermore, we find that TGT can recognize analogues composed entirely of DNA, but only when 2'-deoxyuridines replace the thymines in the DNA. The requirement for uridine bases for recognition is perhaps not surprising given the UGU recognition motif for TGT. However, it is not clear if the uracil requirement is due to specific recognition by TGT or due to the effect of uracils on the conformation of the oligonucleotide.

TGT. However, provided that TGT can position the UGU sequence in the active site in the proper orientation, the UGU sequence need not reside in the anticodon loop to be recognized. Recent studies have shown that TGT can recognize the UGU sequence in at least 2 additional minihelical contexts, at the base of the TΨC stem in yeast tRNA^Ψ^ and in the anticodon position in the absence of U33 (4, 5). Thus, tRNA recognition by TGT is more flexible than previously believed. This observation prompted an examination of the ability of TGT to recognize RNAs containing modifications of the UGU sequence. The initial studies that identified the UGU sequence as the major identity element utilized only canonical base (C, G, A, U) replacements (2, 3). Previous experiments have demonstrated that a DNA analogue of an RNA minihelix corresponding to the anticodon arm of *Escherichia coli* tRNA^Ψ^, ECYM (dECYMH) was inactive. However, there are two fundamental differences between RNA and DNA, the lack of the 2'-hydroxyls and the presence of thymidine rather than uracil in DNA. Therefore, dECYMH has a TGT sequence rather than a UGU sequence.

Given the importance of the UGU sequence in TGT recognition, it is possible that the inactivity of dECYMH was due to the presence of thymidine rather than the loss of the 2'-hydroxyls. To investigate the role of the 2'-hydroxyl in TGT recognition and catalysis, we have studied a deoxyguanosine 34 analogue of ECYM (Fig. 1, dG34ECYMH). This analogue is a substrate for TGT with less than a 10-fold reduction in activity. To further probe the ability of TGT to recognize RNA analogues lacking the 2'-hydroxyl, modified DNA analogues of the previously described minihelix ECYM (2, 6) (Fig. 1, dUdSCFMH(TΨC)) were synthesized and characterized. These analogues (containing deoxyuracil (dU) bases rather than thymidine bases) all serve as substrates for TGT, indicating that the tRNA-guanine transglycosylase from *E. coli* can recognize and modify DNA.

**EXPERIMENTAL PROCEDURES**

*Reagents—*Reagents were purchased from Sigma, Aldrich, or Invitrogen unless otherwise noted. Buffered phenol, glycerol, and HEPES were from United States Biochemical (USB). Tris/HCl buffer was from Research Organics. 8-[1H]Guanine (10 Ci/mmol) was from Moravek Biochemicals. TGT was isolated as previously described (4, 5).

**Synthesis of Minihelical Analogues**—The RNA minihelix, ECYM, was chemically synthesized by automated chemical synthesis performed on an Expedite nucleic acid synthesis system (model 8909, PerSeptive Biosystems) using the manufacturer’s protocols and reagents. However, the RNA phosphoramidite monomers (G, C, A, and U) and CPG columns were from Glen Research. dG34ECYMH was the generous gift of Dr. Houng-Yau Mei (Bioorganic Chemistry, Pfizer Global Research and Development, Ann Arbor, MI). The dU-DNA analogues, dUdECYMH, dUdGU^Ψ^, and dUdSCFMH(TΨC) were from 8-[3H]Guanine (10 Ci/mmol) was from Moravek Biochemicals. TGT was isolated as previously described (4, 5).
TGT Recognizes DNA

| Analogue         | $K_m$ | $k_{cat}$ | $k_{cat}/K_m$ | Relative $k_{cat}/K_m$ |
|------------------|-------|----------|---------------|------------------------|
| ECY              | 3.63  | 4.92     | 1.36          | 1                      |
| ECYMH            | 4.68  | 2.32     | 0.50          | 0.37                   |
| dG$_3$ECYMH      | 1.40  | 0.30     | 0.21          | 0.15                   |
| dUdECYMH         | 6.01  | 0.46     | 0.078         | 0.036                  |
| UGT$_{1-14}$     | 6.22  | 0.20     | 0.032         | 0.024                  |
| dUdUGU$_{1-14}$  | 6.46  | 0.28     | 0.044         | 0.032                  |
| SCFMH(TPC)       | 1.57  | 0.19     | 0.12          | 0.088                  |
| dUdSCFMH(TPC)    | 4.06  | 0.17     | 0.041         | 0.030                  |

$^a$ Standard errors are shown in parentheses. Standard errors for $k_{cat}/K_m$ were calculated as in the following equation.
S.E.($k_{cat}/K_m$) = ($k_{cat}/K_m$) × $\sqrt{\left[\text{S.E.} K_m\right]^2 + \left[\text{S.E.} k_{cat}\right]^2}$

$^b$ Kinetic parameters are determined from the average of two (ECYMH & dG$_3$ECYMH) or three (ECY, dUdECYMH, UGU$_{1-14}$, dUdUGU$_{1-14}$, SCFMH(TPC) & dUdSCFMH(TPC)) replicate determinations of initial velocity data.

RESULTS AND DISCUSSION

The observation that TGT recognition of tRNA is flexible enough to accommodate the UGU sequence in alternate contexts (4, 5) prompted the investigation of the capability of TGT to recognize a modified RNA minihelix. A 2'-deoxyguanosine 34 analogue of ECYMH (dG$_3$ECYMH) was synthesized and shown to be a substrate for TGT with an 8-fold decrease in $k_{cat}$ and a 3-fold decrease in $K_m$ with respect to ECYMH (Table I).

The substantial activity of dG$_3$ECYMH rules out any requisite participation of the 2'-hydroxyl in the chemical mechanism of the TGT reaction. This conclusion is consistent with mutagenesis studies that implicate an aspartic acid residue as the enzymatic nucleophile (9, 10). Thus, it seems that any potential H-bonding interaction between the 2'-hydroxyl of G$_3$4 and TGT is not critical for binding or activity.

To determine the importance of the remaining 2'-hydroxyls, a DNA analogue of ECYMH (dUdECYMH) was synthesized and characterized. This analogue is comprised entirely of 2'-deoxyribonucleotides. However, because of the importance of the UGU sequence in TGT recognition, it contains deoxyuridines in place of thymidine bases. This allows the effect of the ribose backbone to be examined exclusively. In addition to dUdECYMH, the dU-DNA analogues of the alternate substrates UGU$_{1-14}$ (4) (dUdUGU$_{1-14}$) and SCFMH(TPC) (5) (dUdSCFMH(TPC)) (Fig. 1) were also synthesized and evaluated.

Native PAGE band shift experiments demonstrate that all of the dU-DNA analogues bind to TGT (Fig. 2A). This indicates that none of the 2'-hydroxyls are critical for binding to TGT. The RNA analogue with the single deoxyribose substitution, dG$_3$ECYMH, qualitatively exhibited the highest ratio of RNA-bound TGT to free TGT (Fig. 2A, lane 3). It is not clear why the removal of the 2'-hydroxyl of G$_3$4 results in apparently tighter binding than the RNA analogue, ECYMH. Although it is unlikely that the overall conformation of dG$_3$ECYMH differs significantly from ECYMH, it is possible that a local change in the sugar pucker of the G$_3$4 could be responsible for the apparently tighter binding. 2'-deoxyribonucleotides typically favor a C2'-endo(N) sugar pucker, whereas ribonucleotides are frequently found in a C3'-endo(S) conformation (11). It seems likely that the predominantly RNA nature of dG$_3$ECYMH yields a structure that is virtually identical to that of the native RNA substrate for TGT. The almost certain change in conformation at position 34 because of the deoxyguanosine could result in an orientation in the active site that is suboptimal for catalysis, but does not interfere with binding. dUdECYMH and the other dU-DNA analogues do show qualitatively less band
formation of any substrate analogue (RNA or DNA) tested to date, to our knowledge. Although it has not yet been firmly established that the stable complex represents a true mechanistic intermediate (e.g. chemical and kinetic competence of the covalent complex), there is a direct correlation between the formation of this complex and enzymatic activity (5). This correlation is consistent with our findings that all the dU-DNA analogues are substrates for TGT (Fig. 3). In general, the activity of the dU-DNA analogues mirrored their RNA analogues (Table I). For example, dUdECYMH, which was the most active DNA analogue, was derived from the “normal” RNA substrate ECYMH. The reduction in the $k_{cat}$ values for dUdECYMH (5-fold) and dG$^{34}$ECYMH (7-fold) with respect to ECYMH suggests that the orientation of dG$^{34}$ is not optimal for catalysis. The decreased activities of the alternate RNA substrates UGU$^{+1}$ and SCFMH(TΨC) demonstrate that catalysis for these analogues is also affected by suboptimal orientation of the UGU sequence as compared with the normal RNA substrate. The essentially identical $k_{cat}$ values of dUdUGU$^{+1}$ and dUdSCFMH(TΨC), relative to their respective RNA analogues, indicate that the ribose backbone does not significantly aid in any rearrangement that might enhance catalysis. The most prominent result presented in Table I is that removal of all the 2'-hydroxyls has a relatively minor effect (5- to 14-fold reduction) on $k_{cat}$. Despite the small increases in $K_m$, the overall specificities ($k_{cat}/K_m$) are quite comparable to the RNA analogues, especially for the alternate substrates UGU$^{+1}$ and SCFMH(TΨC).

The activity of the dU-DNA analogues can be explained in one of two ways. The first explanation is that TGT is not sensitive to the differences between RNA and DNA. However, we know that this is not strictly accurate because the analogue containing thymidine (dECYMH) is inactive. Yet, replacement of the thymidine bases with deoxyuracil does restore activity with TGT; thus, TGT recognition is not strictly dependent upon the ribose backbone or any conformational effects of the 2'-hydroxyls. The capability of TGT to recognize the UGU sequence in alternate contexts (4, 5) suggests that TGT recognition is fairly indiscriminate. However, recent results from our laboratory (4) show that TGT recognition is blocked when the UGU sequence is locked into certain conformations (e.g. the anticodon loop “U-turn”).

An alternative explanation is that the dU-DNA analogues are able to emulate the RNA analogues despite differences in the preferred conformations of deoxyribonucleotides versus ribonucleotides. The most prevalent (and most recognizable) form of DNA is the B-form double helix in which the sugar conformation is 2'-endo. Because it lacks the 2'-hydroxyls,
DNA can also exist in several different forms, including A-DNA and Z-DNA (11). Conversely, RNA exists predominantly in the A-form with a 3'-endo ribose conformation. Given the greater flexibility of DNA, it is possible that DNA analogues can adopt a conformation that is similar to the corresponding RNA in solution.

Paquette et al. (12) compared the conformation of a tRNA with its "tDNA" and "anti-tDNA" (the complement of tDNA) analogues. By examining the mung bean nuclease cleavage patterns of tRNA\textsubscript{Met}, tDNA\textsubscript{Met}, and anti-tDNA\textsubscript{Met} these authors demonstrated that the global structures of tRNA and tDNA were quite similar, although the core regions of the tDNAs are more exposed than the tRNA. Furthermore, tDNA\textsubscript{Met} and anti-tDNA\textsubscript{Met} are both cleaved by the restriction enzymes HhaI and CfoI, which verifies the presence of base-paired stems. Based on these studies, the authors conclude that nucleic acid conformation is largely determined by the interactions of the bases and those elements common to both DNA and RNA. One role of the 2'-hydroxyls (in tRNA at least) is to increase the stability of the molecule (12). Studies using transition metal complexes have confirmed that the global structure of tDNA\textsubscript{Met} resembles that of tRNA\textsubscript{Met} but that the base-paired stem conformations differ somewhat (13). Those results are most consistent with a more B-like conformation for the tDNA double helical regions. Additionally, Holmes and Hecht have shown that Fe-bleomycin cleaves tRNA\textsubscript{His} and tDNA\textsubscript{His} at the same major site, T\textsuperscript{35} or T\textsuperscript{35} respectively (14). All of the above studies were conducted using canonical base replacements (A, C, G, T, and deoxyribose for the tDNA analogues) and demonstrate that tDNA and tRNA can have similar conformations in solution.

Additional studies have revealed that the presence of modified bases further increases the capability of DNA to mimic RNA (15–18). For example, DNA analogues of the yeast tRNA\textsubscript{Phe} anticodon stem loop containing modifications such as deoxyuracil, 5-methylcytidine (m\textsubscript{5}C), and 1-methylguanine (m\textsubscript{1}G) are able to bind Mg\textsuperscript{2+}. This binding is dependent upon the presence of the modified bases (15). The Mg\textsuperscript{2+} is bound in the upper part of the DNA hairpin (16) in a position that is analogous to that seen in the x-ray crystal structure of yeast tRNA\textsubscript{Phe} (19). Furthermore, the modified DNA analogues were able to bind to poly(U)-programmed 30 S ribosomal subunits and competitively inhibit the binding of native tRNA\textsubscript{Phe} (17). They were also able to inhibit protein expression in a coupled transcription-translation system (18). The solution structure of the fully modified DNA analogue demonstrates that the helix is in the B-form. However, the conformation of the anticodon loop includes the formation of the U-turn and is strikingly similar to that of yeast tRNA\textsubscript{Phe} (18). This demonstrates that the structure of tRNA is not strictly dependent on the ribose backbone and underscores the importance of modified bases in tRNA structure and function.

In light of these experiments, it is perhaps less surprising that tDNA analogues of tRNA can be aminoacylated. \textit{E. coli} tDNA\textsubscript{Phe} (with a 3'-terminal riboadenosine) and \textit{E. coli} tDNA\textsubscript{Lys} were aminoacylated by their respective aminoacyl-

![Fig. 3. Michaelis-Menten plots of the minihelix dU-DNA analogues. A, dG\textsuperscript{34}ECYMH; B, dUdECYMH; C, dUdGU\textsuperscript{1+}; and D, dUdSCFMH(T\textsuperscript{74}C). The averages of data points obtained from three independent determinations are plotted. The curves represent fits of the data calculated by nonlinear regression. Error bars were generated from the standard deviation within each point.](http://www.jbc.org/Content/7181/Figure3.jpg)
tRNA synthetases (20). In a similar fashion, E. coli methionyl-tRNA synthetase will aminoacylate a tDNA^Met analogue (21). Giege et al. (22) have utilized a mutant T7 RNA polymerase to selectively incorporate deoxyribose derivatives of each base (dA, dG, dC, or dU) in order to study the effect of replacing a subset of 2'-hydroxyls. Their results indicate that the yeast methionyl-tRNA synthetase will tolerate dA and dU substitutions, but large decreases in charging occur for dG or dC analogues. Similarly, yeast aspartyl-tRNA synthetase will efficiently charge dC and dA analogues but not dG or dU analogues (22).

The recognition of DNA analogues of RNA substrates also extends to some RNA editing and modifying enzymes. For example, a substrate containing all DNA residues (except for a single ribonucleotide at the cleavage site) can be cleaved by the hammerhead ribozyme, albeit with reduced efficiency (23–25). From a catalytic perspective, a predominately DNA analogue of the ribozyme domain is capable of cleaving an RNA substrate (26). Other examples of RNA catalysis with DNA substrates include Group I introns (27) and RNase P (28, 29). There is also some evidence that other tRNA modification enzymes are capable of recognizing DNA analogues. A tDNA^Met analogue is threonylated to a small degree by a crude yeast extract and is able to inhibit the threonylation of tRNA^Met (21); however, these results need to be verified under more stringent conditions. A dU-containing DNA minihelix analogue of the TΨC stem and loop of yeast tRNA^Met was reported to be a substrate for E. coli m^5U^Ψ4-tRNA methyltransferase (RUMT) (30). This analogue, presumably acting as a weak competitive substrate, was also able to inhibit the methylation of RNA substrates and reduced the aminoacylation of yeast tRNA^Met. Although some activity was seen, this activity was not linear with respect to time. Furthermore, these authors later reported that RNA mutants with single (dU^Ψ4 or dU^Ψ5) or double (dU^Ψ4dU^Ψ5) mutations were not substrates for E. coli RUMT (31). No explanation was given in this later report to account for the activity previously seen with the entirely deoxyribosyl analogue. Therefore, there is some ambiguity as to whether or not RUMT will recognize a DNA analogue. The tRNA editing enzyme that catalyzes the precise addition of the 3'-terminal CCA sequence to tRNAs (the CCA-adding enzyme, ATP:CTP:tRNA nucleotidyl-transferase) will recognize tDNA analogues provided they have a 3'-terminal ribonucleotide (32). Both full-length and minihelix DNA analogues of the TΨC stem and loop of tDNA^Val and tDNA^Met were substrates for the E. coli CCA-adding enzyme. Interestingly, the minihelix analogues were slightly better substrates than the full-length analogues for the CCA-adding enzyme (32).

The activity of the deoxyuridine-containing DNA analogues with E. coli TGT clearly demonstrates that TGT recognition is not critically dependent upon the native ribose backbone. However, the 2'-hydroxyl probably does influence binding, either through conformational effects or direct interactions with TGT. These experiments also demonstrate that TGT is capable of recognizing DNA provided that a UGU sequence can be found. This suggests that under certain conditions (e.g. in the E. coli mutant that lacks the enzymes deoxyribouracil-triphosphatase (dUTPase) and uracil N-glycosylase (E. coli dut)), there may be a physiological role for queuine modification of DNA.

Acknowledgment—We thank members of our laboratory for critical reviews of this manuscript and helpful discussions.

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*J. Biol. Chem.* 2002, 277:7178-7182.
doi: 10.1074/jbc.M111077200 originally published online December 21, 2001

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