tRNA-guanine transglycosylase from *Escherichia coli*: recognition of full-length 'queuine-cognate' tRNAs

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Abstract A key enzyme involved in the incorporation of the modified base queuine into tRNA (position 34) is tRNA-guanine transglycosylase (TGT). Studies of the recognition of truncated tRNAs by the *Escherichia coli* TGT have established a minimal recognition motif involving a minihelix with a 7 base loop containing a U-G-U sequence (where G is replaced with queuine) [Curnow, A.W. and Garcia, G.A. (1995) J. Biol. Chem. 270, 17264–17267; Nakanishi, S. et al. (1994) J. Biol. Chem. 269, 32221–32225]. Still, a clearer understanding of the recognition of full-length 'queuine-cognate' tRNAs by TGT remains lacking. In this paper, we report the in vitro transcription and enzymological characterization (K_m and k_{cat}) of all four 'queuine-cognate' tRNAs from *E. coli* and from *Saccharomyces cerevisiae* with the TGT from *E. coli*. No primary or secondary structures emerge as important recognition elements from this study. The modest differences in substrate specificity (relative k_{cat}/K_m values vary from 0.5 to 8.4) seen among these 'queuine-cognate' tRNAs most likely result from the accumulated effects of many subtle factors. Interestingly, the yeast tRNAs are essentially equivalent to the *E. coli* tRNAs as substrates for TGT, indicating that there is nothing intrinsic to the yeast tRNAs that accounts for the absence of queuine in yeast.

Key words: Queuine; Modified nucleoside; RNA recognition; tRNA

1. Introduction

Queuine (7-(4,5-*cis*-dihydroxy-1-cyclopenten-3-ylaminomethyl)-7-deazaguanine) is a hypermodified base that is found in the wobble position (#34) of the anticodon of four tRNAs (tyrosine, aspartic acid, asparagine, and histidine). In eukaryotes, queuine (a dietary factor for eukaryotes) is incorporated into tRNA via a transglycosylation reaction catalyzed by tRNA-guanine transglycosylase (TGT). In eubacteria (such as *Escherichia coli*), the queuine biosynthetic pathway is more complicated, involving a number of other enzymes in addition to TGT [1]. In *E. coli*, TGT incorporates a queuine precursor (7-aminomethyl)-7-deazaguanine, preQ$_1$) into tRNA. This precursor is then further elaborated to queuine by subsequent enzymes [1]. It has been shown that unfractionated yeast tRNA and *E. coli* G34 tRNAs (tRNA^{Tyr} and tRNA^{Asn}) are substrates for the TGT from *E. coli* [2,3]. Unfractionated *E. coli* tRNA, preQ$_1$- and Q-containing tRNA^{Tyr}, on the other hand, were not substrates. These results indicate that the tRNA substrate for *E. coli* TGT contains guanosine at position 34. The observation that preQ$_1$-34 tRNA^{Tyr} was not a substrate also suggests that the *E. coli* TGT-catalyzed reaction is irreversible. It was later shown that the totally unmodified *E. coli* tRNA^{Tyr} generated by in vitro transcription has identical in vitro kinetic parameters (within experimental error) to those for the Q-deficient (i.e. G34) but otherwise modified *E. coli* tRNA^{Tyr} [4]. This indicates that none of the post-transcriptional modifications except queuine itself plays a significant role in TGT recognition of tRNA. Studies of the recognition of truncated tRNAs by the *E. coli* TGT have established a minimal recognition motif involving a minihelix with a 7 base loop containing a U-G-U sequence (the G is replaced with queuine) [5,6]. While these studies have elucidated a minimal recognition motif, a clearer understanding of the recognition of full-length 'queuine-cognate' tRNAs by TGT is still lacking.

In this paper, we report the in vitro transcription and enzymological characterization (K_m and k_{cat}) of all four 'queuine-cognate' tRNAs from *E. coli* and the corresponding tRNAs from *Saccharomyces cerevisiae* with the TGT from *E. coli*. Even though yeast tRNAs do not naturally contain queuine, the four *S. cerevisiae* tRNAs were included in the study for two reasons. First, to broaden the base of sequence variation from that contained in the four *E. coli* tRNAs alone. Second, while previous research has shown that unfractionated yeast tRNA is an in vitro substrate for the *E. coli* TGT [2], it still remains unknown how well yeast tRNA is recognized by this enzyme. It is possible that there is something about the yeast tRNAs that attenuates their recognition by TGT sufficiently to at least partially account for the absence of queuine in yeast tRNAs. This is, to our knowledge, the first report of the characterization of a complete set of tRNA substrates for any modifying enzyme.

2. Materials and methods

Reagents were purchased from Sigma, Aldrich, and Gibco-BRL unless otherwise noted. Bactotryptone and yeast extract were from Difco Laboratories. Buffers and the Sequenase version 2.0 kit were from United States Biochemicals. Most of the restriction enzymes were from New England Biolabs and Boehringer Mannheim, except for *Eco*T22I which was from United States Biochemicals. Nucleoside triphosphates were from Pharmacia. Oligonucleotides were synthesized at the University of Michigan Biomedical Research Resources Core Facility. Inorganic pyrophosphatase was from Boehringer Mannheim. RNase inhibitor was from Gibco-BRL. 8-[^{14}C]Guanine (56 mCi/mmol) was from Moravek Biochemicals. TGT was isolated from an overexpression clone as described previously [7,8]. T7 RNA polymerase was isolated from an overexpression clone (pAR1219,

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Abbreviations: ECD, ECH, ECN, and ECY, tRNAs Asp, His, Asn, and Tyr from *Escherichia coli*; SCD, SCH, SCN, and SCY, tRNAs Asp, His, Asn, and Tyr from *Saccharomyces cerevisiae*; EDTA, ethylenediamine tetraacetic acid; DTT, dithiothreitol; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid

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obtained from F.W. Studier, Brookhaven National Laboratory) essentially as described by Grodberg and Dunn [9].

2.1. Construction of tRNA in vitro transcription clones

Two PCR primers and oligodeoxynucleotides containing a T7 promoter, various tRNA genes (Asn and Asp from *E. coli* and Asn and Tyr from *S. cerevisiae*), a *Bam*HI site at the 5′ end, and an *Eco*RI site at the 3′ end were used to generate the in vitro transcription clones (sequences available from the author). These oligodeoxynucleotides were PCR amplified using UlTma DNA Polymerase (Perkin Elmer) following the standard protocol provided by the manufacturer. The PCR reaction was performed on either a Genetic Thermal Cycler (Model GTC-1, Precision Scientific, Chicago, IL) or a Perkin-Elmer/Cetus DNA Thermal Cycler (Perkin-Elmer, Norwalk, CT). The reaction conditions were such that the reactants were allowed to denature at 94°C for 1 min, anneal at 45°C for 2 min and then extend at 72°C for 1 min in each cycle for 30 cycles. PCR amplified fragments were extracted with phenol and chloroform and then ethanol precipitated. The pellets were resuspended in 30 μl of double deionized water (ddH_2O).

The PCR products from above were subcloned into pTZ18U (Pharmacia Biotech). 4 μl of the PCR product was double digested with 10 units each of *Bam*HI and *Eco*RI in a 20 μl reaction at 37°C for 2 h and purified by 1% low melting point agarose (SeqPlaque, FMC Bioproducts) gel electrophoresis using the TAE buffer system (40 mM Tris-acetate, 2 mM $Na_2EDTA{\cdot}2H_2O$, pH 8.5). Approximately 0.2 μg of pTZ18U was digested and purified in an identical manner. Gel slices (approximately 50 mg each) containing desired inserts or vector were treated with 1 unit of GELase (Epicentre Technologies) at 45°C for 1 h to digest the agarose into oligosaccharides. The DNA fragments were then ligated using T4 ligase (New England Biolabs) at 16°C overnight following the vendor's protocols. The insert to vector molar ratio is approximately 3 to 1 in a typical ligation reaction. 10 μl of the ligation mixture was transformed into 90 μl of competent *E. coli* TG2 cells the next day following a literature method [10]. Blue/white selection was used to select transformants with inserts in the multiple cloning site (MCS) of pTZ18U. Plasmid preparations from randomly picked white colonies were further screened using restriction enzyme digestions (*Bam*HI and *Afl*III). The sequences of the selected plasmids were confirmed by dideoxy sequencing (performed either in our lab or at the University of Michigan Biomedical Research Resources Core Facility). All of the plasmid preparations used for sequencing were prepared using the QIAprep Spin Column (Qiagen) following the vendor's standard protocol.

2.2. Preparation of template DNAs – plasmid method

Plasmids containing the T7 promoter-tRNA genes were prepared in large scale using an alkaline lysis-PEG precipitation method [11]. The purified plasmid DNAs were then subjected to restriction endonuclease digestion in order to generate the templates for 'run-off' transcriptions as described below.

(i) pECD2, PTFMA, pECN2, pSCN2, ptRNA2, and pSCY2: 200 μg of each plasmid was digested with approximately 50 units *Bst*NI in a 250 μl reaction mixture (NEBuffer 2, New England Biolabs, final concentrations: 10 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT) at 60°C overnight. The reactions were then stopped by phenol/chloroform extraction and the DNAs were recovered by ethanol precipitation at −20°C overnight. The pellets were resuspended in 500 μl of ddH_2O and were ready to be used as templates in the transcription reactions.

(ii) pGFIB/$tRNA^{His}$ and pTFMHis: 200 μg of each plasmid was digested with 50 units of *Eco*T22I in a 250 μl reaction mixture (USB buffer H, USB, final concentrations: 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT) at 37°C overnight. (It was found that incomplete digestion led to multiple transcription products. Overnight digestion ultimately yielded a single, predominant transcription product.) The reactions were stopped by heat inactivation of the enzyme at 65°C for 15 min and then subjected to Klenow fragment treatment (to remove the resultant 3′ overhang) after cooling to room temperature. Approximately 200 units of Klenow fragment (New England Biolabs) were added in 4 ml reaction mixtures in which the concentrations of DNA and Klenow fragment were about 50 μg/ml and 1 unit/μg DNA, respectively, based on the vendor's protocol. The above reaction mixtures containing 10 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, and 7.5 mM DTT were incubated at 30°C for 30 min to 1 h. The reactions were stopped by adding 10 mM EDTA or heating to 75°C for 20 min. The DNA was extracted, ethanol precipitated and resuspended in 500 μl of ddH_2O as described above.

2.3. Preparation of template DNAs – PCR method

DNA fragments containing a T7 promoter and the desired tRNA gene were amplified via PCR from pGFIB/$tRNA^{His}$ and pTFMHis in 100 μl reactions. The PCR reaction conditions were identical to those described above except that the annealing temperature for pGFIB/$tRNA^{His}$ was 45°C and that for pTFMHis was 50°C. Without further manipulation, the PCR products were used as templates in transcription reactions.

2.4. In vitro transcription reaction

500 μl of the resuspended template, generated following the method described in Section 2.2, was added to a 4 ml transcription mixture composed of 40 mM Tris-HCl, pH 8.0, 20 mM $MgCl_2$, 5 mM DTT, 1 mM spermidine, 5 μg/ml BSA, 4 mM each NTP (ATP, CTP, UTP, and GTP), 1 U/ml inorganic pyrophosphatase, 25 U/ml RNase inhibitor, and 250 nM T7 RNA polymerase. This mixture was incubated at 37°C for approximately 4.5 h. Under the above reaction conditions, the template concentration is ca. 50 μg of template/ml transcription mixture. The reaction was stopped by phenol/chloroform extraction and the aqueous layer was ethanol precipitated at −20°C overnight.

The template prepared from the method descibed in Section 2.3 (i.e. 100 μl from one PCR reaction) was added to a transcription mixture of a volume ranging from 1.3 to 2.6 ml, depending on the concentration of the PCR product (estimated from the intensity of the band after 1% agarose gel electrophoresis). The transcription reaction was conducted as described before, and the transcript was phenol/chloroform extracted and ethanol precipitated.

2.5. Purification of transcripts

The transcript pellets were resuspended in 4–8 ml of buffer A (10 mM HEPES, pH 7.4, 1 mM EDTA, and 7 M urea), either filtered through a 0.22 μm sterile filter or centrifuged at 6500 rpm (microcentrifuge) at 4°C to remove any particulate matter. They were then purified via anion exchange chromatography (MonoQ HR10/10, Pharmacia) with a 0–1 M NaCl gradient in degassed buffer A. Fractions containing tRNA (determined by denaturing PAGE, as described below) were pooled.

Pooled eluants from the MonoQ column were desalted using either Centriprep-10 concentrators (molecular weight cutoff: 10 000 Da, Amicon) or a desalting column (Fast Desalting column, HR10/10, Pharmacia) and exchanged into 10 mM HEPES, pH 7.4. Concentrations of tRNAs were determined spectrophotometrically using the extinction coefficients at 260 nm calculated from the base composition of each transcript, corrected for hypochromicity. Approximately 0.1 OD_{260} of each tRNA was incubated with 10 μg of RNase A in 1 ml of 10 mM phosphate buffer, pH 7.0 at 37°C for 1 h. The A_{260}s of undigested and digested RNA were obtained by measuring absorbances prior to the addition of the RNase A and after the reaction was stopped. The determinations of the hypochromicity factors (HCFs) were based on the following equation: HCF = (A_{260} of digested RNA)/(A_{260} of undigested RNA). In all cases HCF was ca. 1.3–1.4, yielding $\varepsilon_{260(\text{corrected})}$ values ranging from 593 to 703 (OD cm^{-1} mM^{-1}).

Preliminary analyses of the desalted tRNAs were conducted using native PAGE. Typically 1–4 μl of tRNAs diluted to 10 mM in 10 mM HEPES were applied to the gel. For the tRNA species that appeared to have more than one conformer, as judged by multiple bands on the gel, renaturation was performed. Otherwise the tRNAs were concentrated by ethanol precipitation and then kinetically evaluated. Renaturation was performed by incubating tRNAs at a concentration of approximately 10–20 μM in 10 mM HEPES, pH 7.4 and 0.5 mM $MgCl_2$ at 68°C for 1 h, cooling immediately to 4°C, and maintaining at 4°C for at least 30 min.

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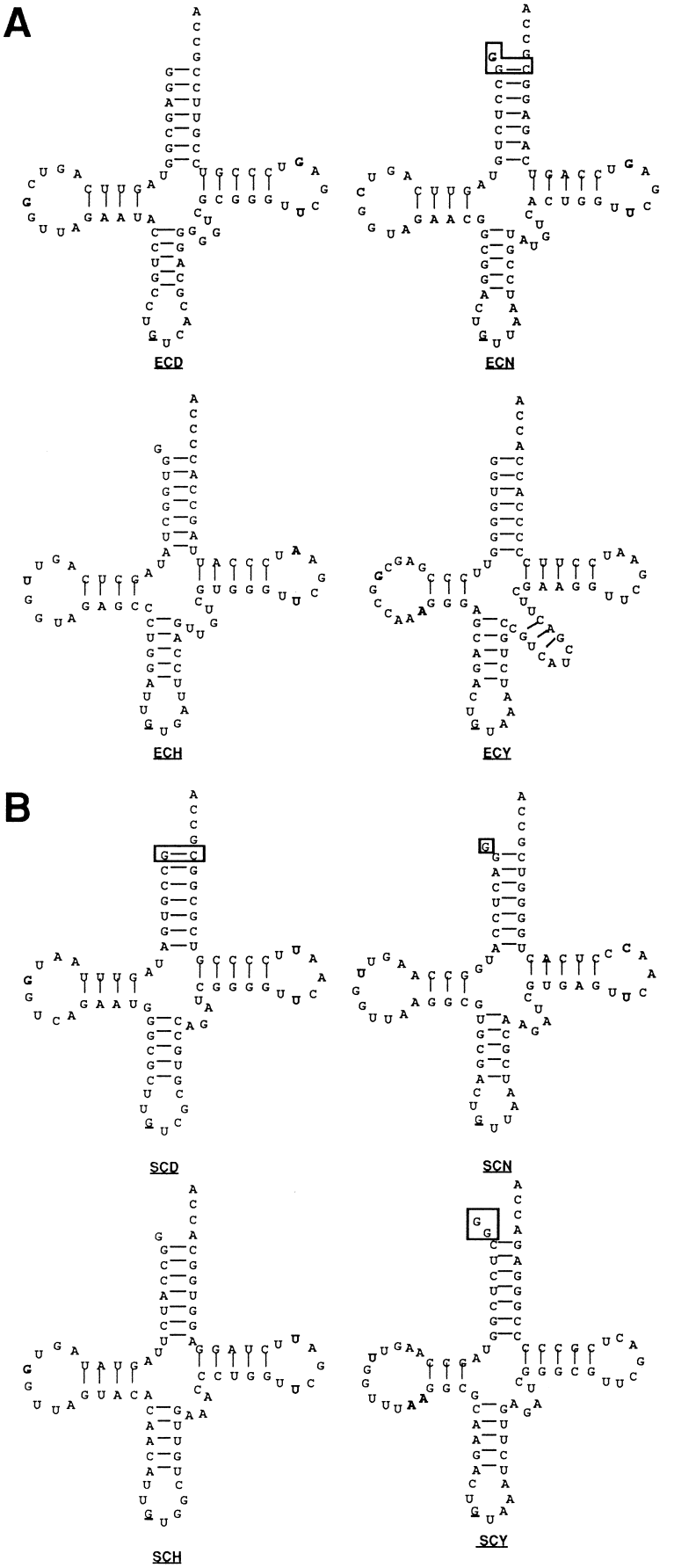
Fig. 1. Full-length unmodified queuine-cognate tRNAs. The base at position 34, where the queuine modification takes place, is underlined. Residues that are different from the natural sequences in order to improve the transcription efficiency are boxed.


A
ECD
ECN
ECH
ECY
B
SCD
SCN
SCH
SCY

2.6. Polyacrylamide gel electrophoresis (PAGE)

Native and denaturing PAGE was performed on a PhastSystem (Pharmacia) as previously described [5,12].

2.7. Band-shift assays

In a typical band-shift assay, 10 μM TGT was first incubated with 2.2-fold excess of full-length tRNAs at 37°C for 15 min in a reaction mixture containing 10 mM HEPES, pH 7.4, 20 mM $MgCl_2$, and 2 mM DTT. The reaction mixtures were then analyzed by native PAGE using Homogeneous 20 polyacrylamide gels (Pharmacia). Two gels were run in parallel, one was stained with ethidium bromide (tRNA visualization) and the other with Coomassie blue (protein visualization).

2.8. Kinetic analyses

A guanine incorporation assay (TGT assay) was used to determine the kinetic parameters (k_{cat} and K_m) for the various tRNAs. In a typical TGT assay, various concentrations of tRNA substrates (ranging from 0.13 to 26 μM for cognate tRNAs) were incubated at 37°C in a reaction mixture composed of 100 mM HEPES, pH 7.4, 20 mM $MgCl_2$, 10 mM DTT, 10 μM 8-[^{14}C]guanine (saturating, K_m ca. 1 μM [4]), and 250 nM TGT. Aliquots (70 μl) were taken at different time points, typically 3, 6, 9, and 12 min after the reaction had been initiated by addition of enzyme, and quenched by acid precipitation in 2 ml of 5% trichloroacetic acid (TCA). The precipitated tRNAs were then collected on glass fiber filters (GF/C filter, Whatman), followed by three TCA washes (ca. 2 ml for each wash) and one ethanol wash. Dried filters were subjected to liquid scintillation counting to determine the level of incorporation of radiolabeled guanine into tRNAs.

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 (Michaelis-Menten plot). Assays were conducted in either duplicate or triplicate. A control experiment, in which the initial velocity for *E. coli* $tRNA^{Tyr}$ at saturating concentration (26 μM) was performed for each assay to normalize the specific activity of TGT from assay to assay. V_{max} and K_m were obtained by non-linear regression of the hyperbolic plots. k_{cat} was obtained by dividing the V_{max} value by the TGT concentration (250 nM).

3. Results

3.1. Preparation of template DNAs – plasmid method

Restriction endonuclease digestion was used to linearize the plasmids to generate the templates for 'run-off' transcriptions. As mentioned earlier, *Bst*NI (CC/WGG, W stands for A or T, the slash indicates the cleavage site) was used in most of the restriction enzyme digestion reactions, except when a *Bst*NI site was present inside the tRNA gene (e.g. ECH and SCH). In the cases when a *Bst*NI site was present inside the gene, *Eco*T22I (ATGCA/T) was used instead. Both *Bst*NI and *Eco*T22I generate the desired GGT sequence at the 5′ end of the template strand. *Eco*T22I digestions, however, result in a 3′ overhang. It is known that the 3′ overhang reduces the yield of in vitro 'run-off' transcriptions. Thus it was necessary to further manipulate the *Eco*T22I digested DNA using an enzyme that has 3′→5′ exonuclease activity, such as *E. coli* DNA polymerase I large fragment (Klenow fragment), to remove the 3′ overhang. Klenow fragment incubations were performed following the vendor's protocols with the exception that only ca. 1/3 unit of Klenow fragment per μg of DNA was used and the DNA concentration was ca. 40 μg/ml.

3.2. Preparation of template DNAs – PCR method

To better control the transcription yield for ECH and SCH, an alternative approach utilizing a PCR amplified fragment containing a T7 promoter and the desired tRNA gene as the transcription template was adopted. The 3′ primer is designed such that the amplified piece would have the correct -CCA 3′ end. As a result, no restriction digestion is necessary prior to the transcription reaction. Approximately 1–2 mg of tRNA can be generated (after purification) using templates from a 100 μl PCR reaction mixture in the transcription reaction.

3.3. In vitro transcription reaction and the purification of transcripts

To improve the yields of the transcription reactions, the gene sequences of $tRNA^{Asn}$ (ECN [13]) from *E. coli* and $tRNA^{Tyr}$ (SCY [14,15]), $tRNA^{Asn}$ (SCN [16]) from *S. cerevisiae* were mutated such that first two bases of the transcripts will be guanines (Fig. 1). These modifications included the simple addition of either one or two extra deoxyguanosine(s) at the 5′ end of the synthetic gene sequence, as in the cases of SCN and SCY. A slightly more complicated modification consisting of both the addition of one extra G at the beginning of the gene and the change of the original U1-A72 base pair in the acceptor stem to G1-C72 was employed in the case of ECN. Note that the in vitro transcription clone for SCD obtained from R. Giegé contains a similar mutation (Fig. 1).

In general, 1–3 mg of purified tRNAs can be obtained from a large scale (usually 4 ml) in vitro transcription reaction. A single species of each transcript for the cognate tRNAs, except the two $tRNA^{Tyr}$s (ECY and SCY), was obtained directly from the pooled anion exchange chromatography eluants. ECY and SCY, purified via anion exchange chromatography, were actually mixtures of dimers and monomers [12,17]. These mixtures can be homogenized into monomers by heating at 68°C for 1 h in the presence of 0.5–1 mM $MgCl_2$. The monomerized tRNAs will stay in the monomer conformation when chilled immediately on ice after monomerization. All of the purified (and monomerized when necessary) tRNAs were suspended in 10 mM HEPES, pH 7.4, and were further diluted

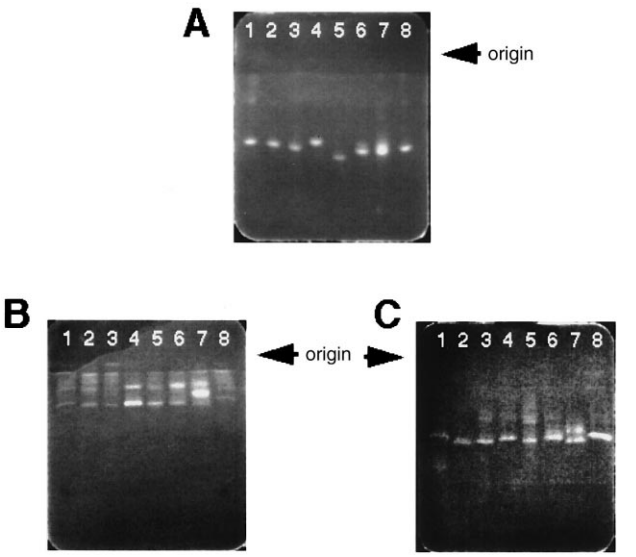


Fig. 2. PAGE of all eight cognate tRNAs. Reaction conditions are described in detail in Section 2. Approximately 5 pmol of tRNA was loaded in each lane. For all three panels: lane 1, ECD; lane 2, ECN; lane 3, ECH; lane 4, ECY; lane 5, SCD; lane 6, SCN; lane 7, SCH; lane 8, SCY. A: Denaturing PAGE, samples were denatured prior to electrophoresis. B: Native PAGE, samples were electrophoresed in the absence of $MgCl_2$. C: Native PAGE samples were incubated at 37°C for 30 min in the presence of 20 mM $MgCl_2$ prior to electrophoresis.

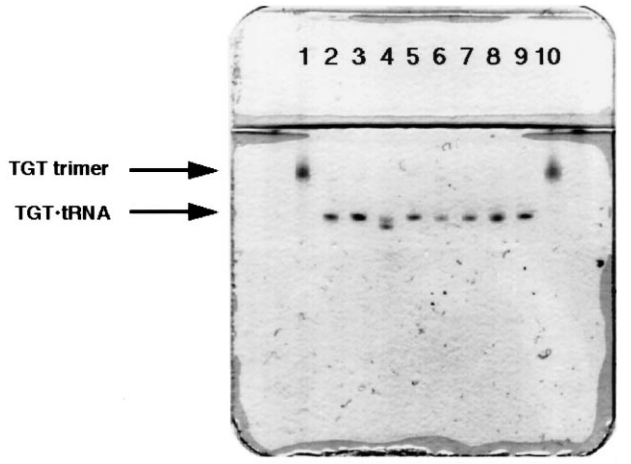


Fig. 3. Native PAGE of TGT and TGT-tRNA complexes. Reaction conditions are described in detail in Section 2. Approximately 0.3 μl sample was loaded in each lane. TGT (10 μM) was preincubated with tRNA (22 μM) and $MgCl_2$ (20 mM) at 37°C for 15 min. Lane 1, TGT alone; lane 2, TGT+ECD; lane 3, TGT+ECN; lane 4, TGT+ECH; lane 5, TGT+ECY; lane 6, TGT+SCD; lane 7, TGT+SCN; lane 8, TGT+SCH; lane 9, TGT+SCY; lane 10, TGT alone.

into different buffer systems for specific studies. The denaturing PAGE shown in Fig. 2A clearly demonstrates that all tRNAs studied in this chapter have been purified to (or very near to) homogeneity. The migration distance of each tRNA is consistent with its size (e.g. ECY, an 85-mer, migrates slower than other tRNAs (ranging from 75 to 80 nucleotides in length), especially the shortest one, SCD (a 75-mer)). Native PAGE analysis of the tRNAs (Fig. 2B) reveals a number of bands for each tRNA. The homogeneity of the tRNAs on denaturing PAGE strongly suggests that these bands are due to different conformers of the tRNAs. This conclusion is supported by the observation that the bands coalesce into essentially single bands for each tRNA when the tRNAs are preincubated in the presence of 20 mM $MgCl_2$ (Fig. 2C). Anecdotal evidence from a number of labs (including ours) suggests that unmodified tRNAs require higher concentrations (ca. 20 mM) of Mg^{2+} for structural stability. All TGT assays (kinetic and band shift) described in this study were conducted in the presence of 20 mM $MgCl_2$.

3.4. Band-shift assays – TGT tRNA interactions

It has been previously shown that, under native PAGE conditions, TGT migrates as a homotrimer [12]. TGT dissociates and forms a complex with tRNA in a monomeric form in the presence of ECY [12] evidenced by a 'band-shift' on native PAGE. A 'band-shift' assay was performed on all eight 'queuine-cognate' tRNAs (Fig. 3). Each of the tRNAs exhibits a qualitatively similar extent of band-shifting under identical conditions.

3.5. Kinetic analyses

Plots of initial velocity versus various tRNA concentrations for the eight cognate tRNAs (not shown) reveal that Michaelis-Menten kinetics are followed for all these tRNAs. The kinetic parameters obtained from non-linear regression of these plots are summarized in Table 1. The relative k_{cat}/K_m values were calculated by dividing the observed value by that for ECY, the first cognate tRNA studied in this lab, to facilitate comparison. These kinetic results suggest that there is no significant difference, in terms of the specificity, among all eight cognate tRNAs except for ECN. Even for ECN, the difference is within one order of magnitude. Both a reduction in K_m over other cognate tRNAs and an increase in k_{cat} contribute to its higher k_{cat}/K_m value.

4. Discussion

Upon comparison of the sequences of the eight 'queuine-cognate' tRNAs studied in this report (see Fig. 1), the only conserved nucleotides, other than those invariant and semi-conserved nucleotides present in all tRNAs, are guanosine 34 and uridine 35. There are several semi-conserved nucleotides located outside the anticodon loop, as depicted in Fig. 4. These semi-conserved nucleotides, however, are not unique to 'queuine-cognate' tRNAs. For example, *E. coli* $tRNA^{Val}$ [18] has a similar set of nucleotide sequences at those positions except that the G30C/U40 base pair present in most of the 'queuine-cognate' tRNAs is replaced by a C30G40 base pair. On the other hand, SCH, which has the most sequence variations at those semi-conserved positions, including a C30G40 base pair, is still a satisfactory substrate for TGT. This simple sequence comparison is clearly insufficient to identify any potential bases involved in TGT recognition of tRNA other than the previously established U-G-U (positions 34–36).

The goal of this report was to experimentally investigate the effect of the differences in primary structure of the different cognate tRNAs on TGT recognition. It was suspected at first that the large variable loop (12 nucleotides) of ECY may have some effect on TGT recognition. Comparison of the kinetic results for ECY with those for SCH, one of the two tRNAs studied that have the smallest variable loops (four nucleotides long), indicates that the variable loop does not play a signifi-

Table 1
Kinetic parameters for several qeuine-cognate tRNAs with the *E. coli* TGT

Analogue	K_m (μM)	k_{cat} (10^{-3} s^{-1})	k_{cat}/K_m (10^{-3} s^{-1} μM^{-1})	Relative k_{cat}/K_m[a]
ECD	1.42 (0.35)	2.48 (0.16)	1.75 (0.54)	1.3
ECH	0.63 (0.17)	2.08 (0.11)	3.27 (1.08)	2.4
ECN	0.69 (0.16)	7.80 (0.38)	11.36 (3.27)	8.4
ECY	3.63 (0.44)	4.92 (0.19)	1.36 (0.22)	1.0
SCD	1.85 (0.42)	6.74 (0.42)	3.65 (1.06)	2.7
SCH	3.60 (1.27)	2.66 (0.30)	0.74 (0.34)	0.5
SCN	1.94 (0.39)	5.08 (0.28)	2.62 (0.67)	1.9
SCY	2.81 (0.33)	4.89 (0.17)	1.74 (0.27)	1.3

Standard errors are shown in parentheses. Kinetic parameters were determined from the average of two (ECD, ECN, SCD, SCH, and SCN) or three (ECH, ECY, and SCY) replicate determinations of initial velocity data.
[a]The relative k_{cat}/K_m value was relative to that for ECY.

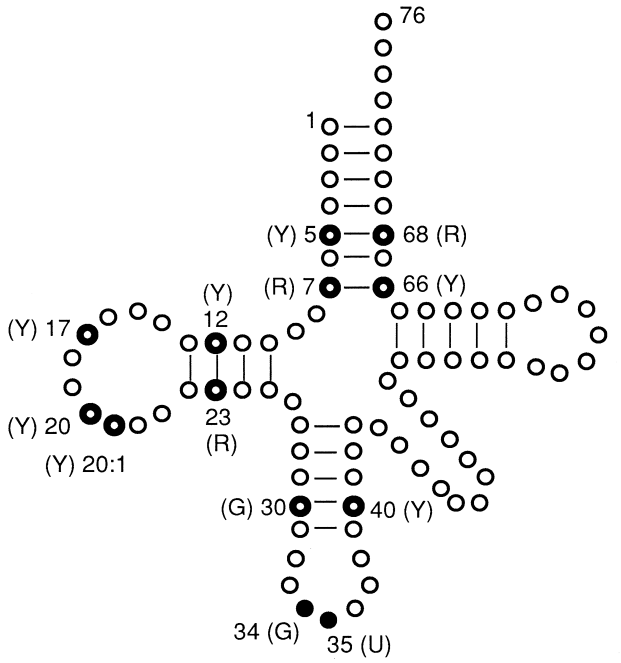


Fig. 4. Conserved and semi-conserved nucleotides present in queuine-cognate tRNAs. Those conserved nucleotides that are common to all tRNAs (e.g., U34, T54, Ψ55) are not included. The invariant and semi-conserved nucleotides are shown in closed and bold circles, respectively. R: purine; Y: pyrimidine.

cant role in TGT recognition. The specificities for these two tRNAs are very similar. However, the specificity for SCD (the other tRNA containing a four nucleotide variable loop) is slightly higher than that for ECY, suggesting that the variable loop size may have some minor effect on TGT recognition.

Results from previous studies have shown that the replacement of G30U40 in a minihelix corresponding to the anticodon stem and loop of the *S. cerevisiae* $tRNA^{Asp}$ (SCDMH) by a canonical base pair, G30C40, resulted in a modest increase in substrate specificity, while the C30G40 double mutation caused a seven-fold loss in specificity [5]. It has been suggested that TGT does not directly interact with the functional groups on the G30U40 base pair. The nature of this base pair, however, may effect the tRNA conformation (or its conformational flexibility) and thus indirectly effect TGT recognition. It is shown clearly in Fig. 1 that SCH is the only cognate tRNA that has a C instead of a G at position 30.

There is a potential conformational change of the anticodon loop of ECH and SCH due to the formation of two canonical/non-canonical intraloop base pairs (i.e. U32A37 and U33G36 for ECH, and U32G37 and U33G36 for SCH [19]). The formation of these two base pairs would result in a two-base turn and greatly restrict the flexibility of the anticodon loop. This kind of loop-closure event would not be seen in the native, fully modified ECH because the modified base present at position 37 can disrupt the formation of the intraloop base pair(s) [19]. The effect of this restricted conformation of anticodon loop is evidently on the catalytic step, as the K_m for ECH is still quite low. One possible explanation for the catalytic effect is that the presumed rigidity of the anticodon loop hinders the tRNA's rearrangement (or fitting) process to the appropriate conformation/orientation for optimal catalysis, which has been proposed by Romier et al. [20], thus resulting in a smaller k_{cat}. Further work is in progress to investigate this.

In summary, there are no primary or secondary structures, other than the previously identified major determinant for TGT recognition (U-G-U), that emerge as important recognition elements from this study. The modest differences in substrate specificity seen among all these 'queuine-cognate' tRNAs most likely result from the accumulated effects of many subtle factors, such as the ones suggested above. These results suggest that each of the 'queuine-cognate' tRNAs should be modified in vivo by TGT to the same extent. Interestingly, the yeast tRNAs are essentially equivalent to the *E. coli* tRNAs as substrates for TGT, indicating that there is nothing intrinsic to the yeast tRNAs that accounts for the absence of queuine in yeast. Studies involving a 'queuine-cognate/non-cognate', chimeric tRNA are in progress in order to further study the role of tRNA structure and sequence on TGT recognition.

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