Regulatory RNAs

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tRNA Shifts the G-quadruplex–Hairpin Conformational Equilibrium in RNA towards the Hairpin Conformer

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Abstract: *Non-coding RNAs play important roles in cellular homeostasis and are involved in many human diseases including cancer. Intermolecular RNA–RNA interactions are the basis for the diverse functions of many non-coding RNAs. Herein, we show how the presence of tRNA influences the equilibrium between hairpin and G-quadruplex conformations in the 5′ untranslated regions of oncogenes and model sequences. Kinetic and equilibrium analyses of the hairpin to G-quadruplex conformational transition of purified RNA as well as during co-transcriptional folding indicate that tRNA significantly shifts the equilibrium toward the hairpin conformer. The enhancement of relative translation efficiency in a reporter gene assay is shown to be due to the tRNA-mediated shift in hairpin-G-quadruplex equilibrium of oncogenic mRNAs. Our findings suggest that tRNA is a possible therapeutic target in diseases in which RNA conformational equilibria is dysregulated.*

Transcripts from three-quarters of the human genome do not encode protein.[1] Emerging evidence of differences in expression levels of non-coding RNAs (ncRNAs) in the context of cellular proliferation processes[2] suggests that these ncRNAs play crucial roles in normal development and disease.[3] Although the functions of the vast majority of ncRNAs are unknown, certain ncRNAs clearly use RNA–RNA interactions to achieve their diverse functions.[4] Hybridization-based approaches have been used to capture the targets of ncRNAs;[5] however, due to the complexity of the cellular environment, which causes nonspecific and off-target hybridization, these approaches do not provide information on how individual ncRNAs influence the functions of their target molecules. One possible way to gain insight into these mechanisms is to identify the influence of ncRNA′s interaction with its target RNA molecule alone. Herein, we focus on transfer RNA (tRNA) as a cellular abundant ncRNA, and investigated its effect on the conformational dynamics of other RNAs, which are able to adopt two mutually exclusive conformations.

tRNA is one of the most abundant ncRNAs, accounting for about 15% of total cellular transcripts.[6] In addition to their key roles in protein synthesis, tRNAs are involved in regulation of gene expression[7] and apoptosis,[8] and can alter the conformation and solubility of proteins.[9] Furthermore, certain tRNAs, such as $tRNA^{Gly}$ (anticodon CCC) and $tRNA^{Arg}$ (anticodon CCU), are overexpressed in cancer cells[10] under control of oncogenic signaling pathways,[11] implicating tRNAs in the proliferation of cancer cells. It is possible that certain tRNAs are responsible for regulation of conformational changes in other cellular RNAs. Well-characterized conformational switches in mRNAs regulate translation efficiency.[12] Conformational equilibria between G-quadruplexes (G4s) and hairpins have been identified in many therapeutically important mRNA sequences.[13–15] The G4-hairpin conformational switch may be involved in neurodegenerative diseases, diabetes, and cancer as dysfunction in these switches can result in aberrant RNA transcription,[13] altered mRNA splicing,[14] and inhibition of Dicer-mediated miRNA maturation.[15] In addition, G4s in 5′ untranslated regions (UTRs) of oncogenic mRNAs affect protein expression levels by suppressing translation.[16] We envisioned that tRNA might affect the conformational dynamics between G4 and hairpin structures through RNA–RNA interactions, ultimately impacting cellular proliferation.

To study the equilibrium between G4 and hairpin structures, we designed two RNA sequences HpG4-1 and HpG4-2, which have ability to form mutually exclusive hairpin and G4 structures (Figure 1). HpG4-1 is an previously designed sequence,[17] and HpG4-2 is a natural sequence derived from *E. coli* eutE mRNA.[18] These two oligonucleotides form hairpin conformers of different stabilities as predicted by Mfold.[19] The hairpin formed by HpG4-2 (predicted $\Delta G° = -11.3$ kcal mol^{-1}) is more stable than that formed by HpG4-1 ($\Delta G° = -9.7$ kcal mol^{-1}). The conformations of these oligonucleotides in the absence and presence of

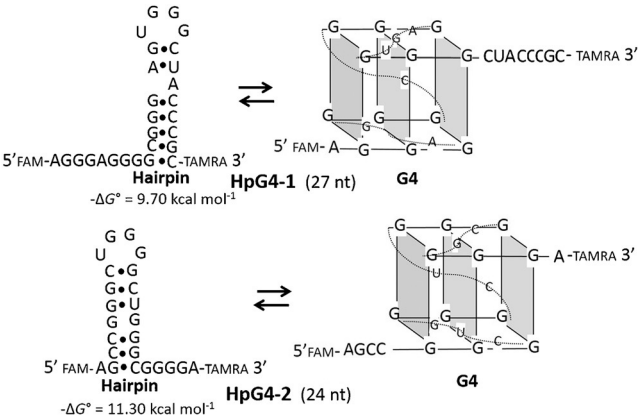

Figure 1. Predicted hairpin and G4 equilibria of sequences used in this study (FAM = 6-carboxyfluorescein; TAMRA = tetramethylrhodamine).

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potassium or lithium ion were analyzed by circular dichroism (CD; Figure S1 in the Supporting Information). In the absence of added cations and in the presence of 100 mM LiCl, spectra of both oligonucleotides showed negative and positive peaks at 210 nm and 260 nm, respectively, characteristic of the A-form RNA helix. In the presence of 100 mM KCl, CD spectra had positive peaks at 210 and 260 nm and a negative peak at 240 nm, indicative of parallel G-quadruplex. These observations suggest that the potassium ion triggers the transition from hairpin to G4.

To evaluate the kinetics and equilibria of the hairpin-G4 conformational transitions by fluorescence resonance energy transfer (FRET), oligonucleotides were labeled with 6-carboxyfluorescein (FAM) and tetramethylrhodamine (TAMRA) at 5′ and 3′ ends, respectively. FRET has been used to study the structure and dynamics of nucleic acids including duplexes [20] and G4s.[21] The conformational transition from hairpin to a relatively compact G4 structure results in an increase of FRET signal (Figure 2a).

We studied the transition kinetics of fluorescently labeled HpG4 oligonucleotides in the absence of tRNA as a function of time after addition of KCl (final concentration 100 mM).

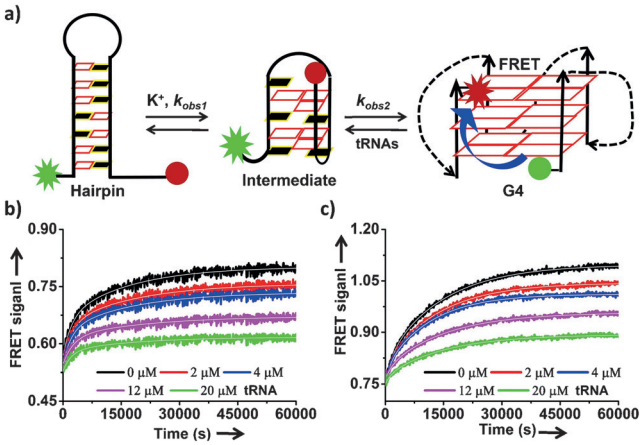

Figure 2. a) Representation of the proposed two-step folding mechanisms and origin of the FRET signal. The intermediate is an ensemble of conformations. FAM (green) and TAMRA (red). b),c) Fluorescence as a function of time after addition of KCl and the indicated concentration of tRNA to b) HpG4-1 and c) HpG4-2. The FRET curves are averages of triplicate experiments. Experiments were performed in a buffer containing 50 mM MES (pH 7.0), 2 mM $MgCl_2$, and 0.05% Tween20 at 37°C. Experiments were initiated by addition of a buffer containing 100 mM KCl with 0 (black), 2 (red), 4 (blue), 12 (purple), or 20 μM (green) tRNA.

Hairpin conformations were pre-formed by refolding the oligonucleotides in the presence of 2 mM $MgCl_2$. FRET signals were monitored during incubation at 37°C (Figures 2b,c); details of the FRET measurement are given in Supporting Information. The increase of FRET signal with increasing the incubation time suggested that there were transitions from the hairpin to the G4 conformer in both oligonucleotides. The obtained time courses of FRET signal changes were fit to a double-exponential rate equation (Supporting Information Equation 1) since fitting to a single exponential rate equation resulted in biased distribution of deviations (Figure S2). The better fit of the double-exponential rate equation is suggestive of an intermediate state in the conformational transition, which is consistent with previously observed triplex-like structure intermediate during G4 folding (as illustrated schematically in Figure 2a).[22]

Table 1 shows the observed rate constants for the first kinetic step (k_{obs1}) and the second kinetic step (k_{obs2}). In the absence of tRNA, both k_{obs1} and k_{obs2} values obtained from both HpG4-1 and HpG4-2 were significantly smaller than previously observed values for an RNA G4 with two quartets that does not form a competitive hairpin structure (k_{obs1} and k_{obs2} were $1.24\times10^2\ s^{-1}$ and $0.18\times10^2\ s^{-1}$, respectively).[22] On the other hand, DNA G4 folding, which has potential to form the competitive hairpin structure,[23] revealed rates similar to those observed here. Both k_{obs1} and k_{obs2} values obtained from HpG4-1 and HpG4-2 were in similar time frames (within experimental error) despite Mfold shows differences in hairpin stabilities. This suggests that the stability of the hairpin conformer does not solely determine the rate of the conformational transition. Both dissociation rate of the hairpin and folding rate of the G4 are likely important.

To evaluate the effect of tRNA on hairpin-G4 transition kinetics and equilibria, the conformational transition experiments were performed in the presence of yeast tRNA ranging in concentration from 2 to 20 μM (Figures 2b,c). FRET signals increased in the presence of tRNA with increasing incubation time. The k_{obs1} and k_{obs2} values were not affected by the presence of tRNA (Table 1), whereas the FRET signal intensities at the saturation points were significantly decreased as the tRNA concentration was increased (Figures 2b,c). The presence of excess non-fluorescent nucleic acid did not affect the FRET signal during G4 folding.[21] Thus, the decrease in FRET signal intensity as a function of tRNA concentration suggests that the tRNA shifts the hairpin-G4 equilibrium toward the hairpin conformer. Notably, when we added tRNA to a sample of HpG4, in which G4 conformer was pre-formed in the presence of KCl, we observed that the

Table 1: Kinetic parameters for hairpin to G-quadruplex transition in the presence of various concentrations of tRNA at 37°C.[a]

Amount of tRNA [$\times10^{-6}$ M]	HpG4-1 k_{obs1} [$\times10^{-4}\ s^{-1}$]	k_{obs2} [$\times10^{-4}\ s^{-1}$]	K_F	G4: hairpin ratio	HpG4-2 k_{obs1} [$\times10^{-4}\ s^{-1}$]	k_{obs2} [$\times10^{-4}\ s^{-1}$]	K_F	G4: hairpin ratio
0	4.78 ± 0.29	0.60 ± 0.04	–	100:0	5.34 ± 1.07	0.67 ± 0.03	–	100:0
2	4.16 ± 0.24	0.51 ± 0.06	6.76	87.1:12.9	7.63 ± 0.92	0.75 ± 0.04	6.82	87.2:12.8
4	4.77 ± 0.36	0.58 ± 0.06	3.99	80.0:20.0	8.41 ± 0.93	0.83 ± 0.04	3.89	79.5:20.5
12	3.71 ± 0.60	0.42 ± 0.02	1.17	53.9:46.1	6.37 ± 2.78	0.65 ± 0.03	1.33	57.1:42.9
20	4.94 ± 0.69	0.56 ± 0.12	0.62	38.4:61.6	6.50 ± 1.55	0.66 ± 0.04	0.66	39.6:60.4

[a] k_{obs1} and k_{obs2} are averages from three experiments, and the errors are standard deviations. K_F was calculated as shown in Supporting Information.

FRET signals inversely decreased with increasing the concentration of tRNA (Figure S3). These results also indicate that equilibrium shifts in favor of hairpin conformer in the presence of tRNA due to interaction between HpG4 oligonucleotides and tRNA, which was confirmed by fluorescence correlation spectroscopy (FCS); the diffusion times of the HpG4 oligonucleotides in the presence of tRNA increased by about 1.5- to 2-fold relative to that in the absence (Figure S4).

The equilibrium constant (K_F) of the hairpin and the G4 conformers at each tRNA concentration was determined using a reversible two-state equilibrium folding model, which assumes that the intermediate structure does not significantly affect FRET signal at equilibrium and that conversion of hairpin to G4 conformer is complete at 100 mM KCl (Supporting Information Equations 2–4). The K_F value decreased with increasing tRNA concentration for both HpG4-1 and HpG4-2 (Table 1). In the presence of 20 μM tRNA, the hairpin conformer predominated with K_F values of 0.62 for HpG4-1 and of 0.66 for HpG4-2. The plots of ln (K_F) versus ln [tRNA] showed linear correlations with slopes of approximately −1 (Figure S5), suggesting that one molecule of tRNA interacts with each HpG4 oligonucleotide.[24] In addition, the equilibrium dissociation constant (K_D) for the interaction between tRNA and HpG4-1 RNA at 37 °C, which was calculated from the FRET signal change depending on the tRNA concentrations (Figure S6), was 22.5 μM with the Hill coefficient value of 0.92. The result supported 1:1 binding between the tRNA and HpG4 RNAs and suggested the concentration range of the tRNA used in this study is relevant. Moreover, the range of tRNA concentration in a typical mammalian cell having 20–30 μm diameter can be calculated to be 8.5–30 μM, which corresponds to the K_D value, based on the estimation that about 15% of total cellular transcripts (10–30 pg in a single cell)[25] are tRNA.[6]

To further shed light on the type of interaction between the tRNA and HpG4 oligonucleotides, we synthesized three RNA hairpins, Hp-1, Hp-2, and Hp-3, that mimic anticodon arm of tRNA (Figures S7a–c). The conformational transitions of HpG4-1 in the presence of 0 and 20 μM anticodon arm mimics showed decreases in FRET signal in the presence of Hp-1 but not in the presence of Hp-2 or Hp-3 (Figure S7d). The hairpin structures can interact with other hairpins by both intramolecular and intermolecular loop-loop interactions.[26] In addition, molecules that interact with single-stranded guanine runs destabilize G4 structures.[27] Thus, these results suggest that the CCC trinucleotides in the loop-region of Hp-1, in which the anticodon trinucleotides have orientations similar to those in the natural tRNA due to the conserved loop sequence, interact with the complementary GGG nucleotides in the single-stranded region of the hairpin conformers of HpG4 oligonucleotides (Figure S8) resulting in the equilibrium shift observed. The small decrease of FRET signal in the presence of Hp-3 compared to Hp-1 also suggests that the pre-organization of the anticodon trinucleotides in the loop-region are required for the efficient loop-loop interaction through proper stacking and orientation. Based on these results, we reasoned that other candidates for interactions are the tRNAs with anticodon loops consisting of mixtures of cytosine and uracil. Uracil potentially forms a wobble base pair with guanine. These candidates are the arginine tRNAs (CCU, UUC) and the glutamine tRNA (CUU).

In vivo, RNA folds during transcription in a process known as co-transcriptional folding. The sequences in the 5′ region may form metastable structures. Based on our in vitro results, we expected that the tRNA might affect co-transcriptional G4 folding if there is a mutually exclusive hairpin conformer.[28] We selected six HpG4 sequences, HpG4-3 to HpG4-8 (Table S1) with the potential to form both hairpin and G4 conformers (Figures S9–S14) from the 5′ UTRs of cancer-related genes. For each of these genes, formation of G4 suppresses protein expression.[16,29,30] DNA templates for transcription of the HpG4 sequences were prepared following our previous work (Figure S15).[28] Transcription was performed at 37 °C in the presence of *N*-methyl mesoporphyrin (NMM), which enables real-time fluorometric monitoring of G4 formation (Figure S16). Co-transcriptional G4 folding and subsequent post-transcriptional G4 formation after addition of DNase were monitored in the presence and absence of 20 μM tRNA.

For all templates, NMM fluorescence signals increased with increasing incubation time both in the absence and presence of tRNA at 37 °C. In the presence of 20 μM tRNA, the signal intensity was significantly lower for HpG4 transcripts with the exception of HpG4-8 (Figure 3 and Figure S17). Nearly the same time course of the fluorescence increases, which were observed with HpG4-8 transcript in the absence and presence of tRNA, suggests that the tRNA has little effect on NMM fluorescence. Thus, the decreased signals with other HpG4 transcripts in the presence of tRNA are due to the shift in equilibria from G4 to hairpin conformer rather than reduction in RNA transcript quantity. The hairpin conformer of the HpG4-8 has the lowest predicted stability of those analyzed. In addition, HpG4-8 potentially forms an alternative hairpin structure that is not mutually exclusive with the G4 conformer (Figure S18). HpG4-8 probably folded

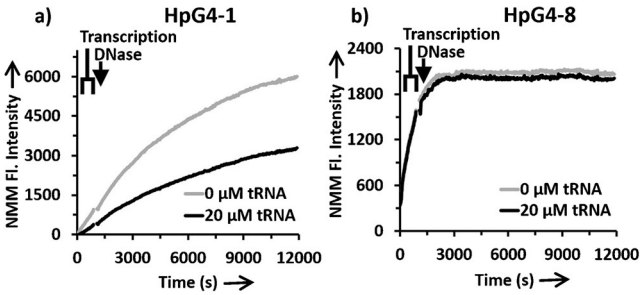

Figure 3. Effect of tRNA on co-transcriptional G4 formation. Representative NMM fluorescence curves at 37 °C for transcription reaction of DNA template for a) HpG4-1, and b) HpG4-8 in the presence of 0 (gray) and 20 μM (black) tRNA. Transcription reactions were performed in a 40 μL buffer containing 50 mM HEPES-LiOH (pH 7.6), 5 mM magnesium acetate, 100 mM potassium glutamate, 2 mM spermidine, 0.05% Tween 20, 5 μM NMM, 1 mM each rNTP, 50 nM DNA template, and 1 U μL^{-1} T7 RNA polymerase. Transcription was halted at 15 min by addition of 2 U DNase (arrow). The NMM fluorescence curves are average of three experiments.

into the G4 conformer during transcription irrespective of the presence of tRNA.

We further evaluated the influence of tRNA on the reporter gene translation. All eight HpG4 (HpG4-1 to HpG4-8) sequences (Figure 1 and Table S1) were cloned into the 5′ UTR of the *Renilla luciferase* reporter gene (Figure S19). We also used a control construct, which has a 5′ UTR sequence consisting of similar length with the HpG4 sequences but cannot form a hairpin or a G4 structure. The reporter mRNAs, which were generated by in vitro transcription with T7 RNA polymerase in the presence or absence of 20 μM tRNAs, were refolded, and subsequently translated using a rabbit reticulocyte lysate system. The *Renilla* Luciferase signals translated from the HpG4 mRNAs are shown relative to that from the control mRNA without HpG4 (Figure 4). In the absence of tRNAs, the luminescence signal for the control

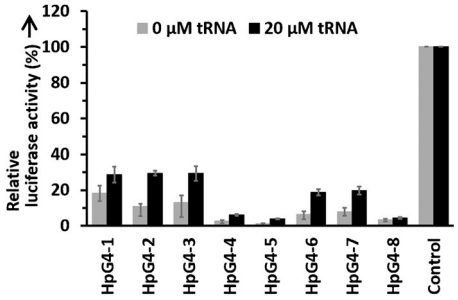

Figure 4. Enhancement of in vitro translation efficiency of reporter mRNAs in the presence of tRNA. Relative luminescence signals obtained from each reporter mRNA with the indicated HpG4 sequence in the 5′ UTR was normalized by that obtained from control mRNA. Error bars represent the standard deviation of five independent experiments.

mRNA was considerably higher than those for the HpG4 mRNAs. The observed suppression of reporter gene translation in HpG4 mRNAs is due to formation of G4 conformer in the 5′ UTR.[16a] In the presence of 20 μM tRNAs, the relative luminescence signals of the HpG4 mRNAs, with the exception of HpG4-8 mRNA, were 1.6- to 3.1-fold higher than in the absence of tRNA. Similar amounts of mRNA transcripts were produced in the presence and absence of tRNAs (Figure S20), and there was a reduction in NMM fluorescence (Figure S21) in the presence of tRNAs, clearly indicating that the observed translation enhancements were due to shifts in hairpin-G4 equilibrium.

In summary, we have investigated the effect of a high concentration of tRNA, which mimics the overexpression of tRNA in certain cancers,[10,11] on the structures and functions of mRNAs able to form mutually exclusive hairpin or G-quadruplex structures. The tRNA shifted the hairpin-G4 equilibrium toward hairpin conformers in synthetic constructs and in those containing 5′ UTR regions of oncogenes and affected co-transcriptional G4 formation and translation efficiency. The cause of the equilibrium shift is likely due to interactions between a guanine run in the mRNA and the CCC anticodon loop of tRNA. It was suggested that the G4 in the 5′ UTR of oncogenes inhibits translation and mutations in the G4 structures may lead to oncogenesis.[16a] Our results suggest that the increased tRNA levels in cancer cells may increase the translation efficiency of oncogenes mediated by the shifting in the hairpin-G4 equilibrium. Moreover, tRNA-derived RNA fragments (tRFs) are also enriched in cancer cells,[31] and emerging evidence suggests that tRFs have functional roles in gene regulation.[7] tRFs having complementary loop sequences might influence the hairpin–G4 equilibrium and translation efficiency as was shown herein for tRNA. Our data indicate that tRNAs should be considered as potential therapeutic targets for treating cancer. Furthermore, our results provide a strategy for testing of the effect of other abundant ncRNAs on conformations of target nucleic acids that will provide insights into functions and mechanisms of ncRNAs.

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