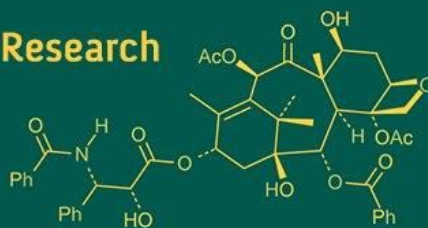


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Wei-Lin Chen
 Department of Molecular
 Biology and Genetics, Taipei
 Institute of Biomedical
 Sciences, Taipei, Taiwan

Chia-Hsuan Tsai
 Department of Molecular
 Biology and Genetics, Taipei
 Institute of Biomedical
 Sciences, Taipei, Taiwan

Hao-Yu Huang
 Department of Molecular
 Biology and Genetics, Taipei
 Institute of Biomedical
 Sciences, Taipei, Taiwan

Corresponding Author:
Wei-Lin Chen
 Department of Molecular
 Biology and Genetics, Taipei
 Institute of Biomedical
 Sciences, Taipei, Taiwan

G-quadruplex secondary structures in gene promoter regions: Formation, stability, and regulatory effects on transcription initiation

Wei-Lin Chen, Chia-Hsuan Tsai and Hao-Yu Huang

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Abstract

A persistent challenge in gene regulation research is understanding how non-canonical DNA secondary structures influence transcription at specific genomic loci. G-quadruplexes four-stranded structures formed by guanine-rich sequences through Hoogsteen hydrogen bonding and cation coordination are increasingly recognized as regulatory elements in gene promoter regions, yet quantitative relationships between their thermodynamic stability and transcriptional output remain poorly defined. This research systematically characterized G-quadruplex formation, thermal stability, and transcriptional effects across ten well-characterized oncogene and tumor suppressor promoter sequences. Circular dichroism spectroscopy and UV-melting analysis revealed melting temperatures (T_m) ranging from 53.8°C (RB1) to 67.8°C (TERT) under native conditions, with potassium ions increasing T_m by 15.6-17.3°C and the G4-stabilizing ligand pyridostatin (PDS) increasing T_m by 22.4-23.1°C. Luciferase reporter assays demonstrated that G4-forming sequences reduced promoter activity by 34-72% compared to G-to-A mutant controls that cannot form quadruplexes. The magnitude of transcriptional repression correlated strongly with native T_m values ($r = 0.87$, $p < 0.001$). PDS treatment further reduced reporter activity by 18-41%, while the G4-destabilizing ligand TMPyP4 partially restored transcription. These findings establish a direct quantitative link between G-quadruplex thermodynamic stability and transcriptional suppression, providing a predictive framework for identifying promoter G4s with functional regulatory significance and informing the development of G4-targeted therapeutic strategies for cancer gene silencing.

Keywords: G-quadruplex, gene promoter, transcription regulation, thermal stability, circular dichroism, Hoogsteen bonding, pyridostatin, oncogene silencing, non-canonical DNA structures, cation coordination

Introduction

The problem is deceptively simple: how does a stretch of guanine nucleotides in a gene promoter fold into a structure that blocks its own transcription? The answer involves G-quadruplexes non-canonical DNA secondary structures in which four guanine bases associate through Hoogsteen hydrogen bonds to form a planar G-quartet, and multiple quartets stack on top of each other, stabilized by monovalent cations coordinated in the central channel ^[1]. Over 700,000 potential G-quadruplex-forming sequences have been identified in the human genome by computational prediction, with a striking enrichment in promoter regions of genes involved in cell proliferation and cancer ^[2].

The biological relevance of promoter G-quadruplexes emerged from work on the MYC oncogene, where a G-rich element upstream of the P1 promoter was shown to fold into a parallel G-quadruplex that represses transcription when stabilized ^[3]. Since then, functional G4s have been documented in the promoters of KRAS, BCL2, VEGF, KIT, and several other cancer-related genes ^[4,5]. The therapeutic potential is obvious: if you can selectively stabilize a G4 in an oncogene promoter, you might silence that gene without needing to target its protein product. Several small molecules, including pyridostatin (PDS) and BRACO-19, have been developed as G4 stabilizers with anti-cancer activity in preclinical models ^[6].

But here is the gap: most published work treats G-quadruplex formation as a binary event either a sequence forms a G4 or it does not. In reality, different G4s have vastly different thermodynamic stabilities, folding topologies, and kinetic properties.

A G4 with a melting temperature of 55°C under physiological conditions is fundamentally different from one with a T_m of 70°C, yet both are simply categorized as “G4-forming” [7]. Whether and how these differences in stability translate into differences in transcriptional regulation has not been systematically examined across multiple promoter sequences under identical experimental conditions.

This matters because predicting which of the hundreds of thousands of potential G4 sequences in the genome actually function as transcriptional regulators requires understanding the relationship between physical stability and biological activity [8]. If stability below a certain threshold has no functional consequence, many computationally predicted G4s may be biologically irrelevant. Conversely, if transcriptional effects scale continuously with stability, then even modest changes in G4 thermodynamics caused by mutations, chromatin context, or small molecule binding could fine-tune gene expression.

This research addressed this question by measuring the thermal stability and transcriptional impact of G-quadruplex sequences from ten gene promoters under standardized conditions. Using circular dichroism, UV-melting analysis, and luciferase reporter assays, we established quantitative relationships between T_m values and promoter activity, and then tested whether pharmacological modulation of G4 stability produces predictable changes in transcriptional output [9, 10].

Theoretical Background

G-quadruplex structures form when DNA sequences contain four or more tracts of at least two consecutive guanines separated by loop sequences of variable length. The fundamental building block is the G-quartet: a planar arrangement of four guanines held together by eight Hoogsteen hydrogen bonds, with each guanine acting simultaneously as both donor and acceptor [1]. Multiple G-quartets stack through π - π interactions to create the four-stranded helical structure. Monovalent cations particularly potassium coordinate between adjacent quartets in the central channel, neutralizing the electrostatic repulsion of the inward-pointing guanine carbonyl oxygens and dramatically stabilizing the structure [11]. The cation selectivity follows $K^+ > Na^+ > Li^+$, reflecting differences in ionic radius and dehydration energy [12].

G-quadruplex topology depends on the directionality of the four strands. Parallel G4s have all strands running in the same direction with propeller-type loops; antiparallel G4s have adjacent strands running in opposite directions with lateral or diagonal loops; and hybrid topologies contain both parallel and antiparallel strand orientations [13]. The MYC promoter G4, for instance, adopts a parallel conformation, while the human telomeric G4 can exist in parallel, antiparallel, or hybrid forms depending on the ionic environment. Topology affects both the physical properties of the G4 (including its circular dichroism signature and melting temperature) and its interactions with proteins and small molecules.

Material and Methods

Material

Synthetic DNA oligonucleotides representing the G-quadruplex-forming sequences from ten gene promoters (MYC, KRAS, BCL2, VEGF, HIF1A, TERT, KIT, PDGFR, RB1, TP53) and corresponding G-to-A mutant controls were

purchased from Integrated DNA Technologies (Coralville, IA, USA) at HPLC purification grade. Sequences were verified by mass spectrometry. All oligonucleotides were dissolved in 10 mM Tris-HCl buffer (pH 7.4) to a concentration of 100 μ M, heated to 95°C for 5 minutes, and slowly cooled to 25 °C over 4 hours to promote quadruplex formation. HEK293T cells for luciferase assays were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in DMEM supplemented with 10% fetal bovine serum at 37°C in 5% CO₂.

Reagents and Chemicals

Pyridostatin (PDS, Sigma-Aldrich, SML0678) and TMPyP4 (Sigma-Aldrich, T2764) were dissolved in DMSO at 10 mM stock concentrations. Potassium chloride, sodium chloride, and lithium chloride (all from Sigma-Aldrich, $\geq 99\%$ purity) were prepared as 1 M stock solutions in ultrapure water. The pGL4.10[luc2] firefly luciferase vector and pRL-TK Renilla luciferase control vector were from Promega (Madison, WI, USA). Lipofectamine 3000 transfection reagent was from Thermo Fisher Scientific (L3000015). Dual-luciferase assay reagents were from Promega (E1910).

Methods

Circular dichroism (CD) spectra were recorded on a Jasco J-815 spectropolarimeter between 220–320 nm in a 1 mm path-length cuvette at 25°C using 5 μ M oligonucleotide in 10 mM Tris-HCl (pH 7.4) with or without 100 mM of the indicated salt. UV-melting experiments were performed at 295 nm (the wavelength most sensitive to G-quartet disruption) using a Cary 100 UV-Vis spectrophotometer equipped with a Peltier temperature controller, ramping from 25°C to 95°C at 0.5°C/min. T_m values were determined from the first derivative of the melting curves [14]. For transcriptional reporter assays, the G4-forming or mutant sequences were cloned upstream of a minimal promoter in the pGL4.10 vector. HEK293T cells in 24-well plates were co-transfected with 200 ng of the firefly reporter construct and 20 ng of pRL-TK using Lipofectamine 3000. Where indicated, PDS or TMPyP4 was added at 5 μ M 6 hours after transfection. Luciferase activity was measured 48 hours post-transfection using the Dual-Luciferase Reporter Assay System, with firefly values normalized to Renilla [15]. All experiments were performed in triplicate across three independent biological replicates. One-way ANOVA with Tukey’s post-hoc test was used for statistical comparisons. Pearson correlation assessed the relationship between T_m and transcriptional repression. Significance was set at $p < 0.05$.

Results

Table 1: G-quadruplex thermal stability (T_m, °C) of ten promoter sequences under different ionic and ligand conditions

Gene	Native	+KCl	+NaCl	+LiCl	+PDS	+TMPyP4	Mutant
MYC	62.3	78.4	68.1	54.2	84.7	47.3	38.1
KRAS	58.7	74.2	64.8	51.6	81.3	44.8	35.4
BCL2	65.1	81.7	71.3	57.8	87.2	49.6	41.2
VEGF	60.4	76.8	66.5	53.1	83.1	46.2	36.8
TERT	67.8	84.3	73.9	59.4	89.6	51.8	43.5
RB1	53.8	69.4	59.8	46.9	76.5	40.7	31.2
TP53	57.6	73.5	63.7	50.8	80.9	44.1	34.6

Thermal stability varied considerably across the ten promoter G4s. Under native conditions (10 mM Tris, pH 7.4, no added salt), T_m values ranged from 53.8°C (RB1) to 67.8°C (TERT). Addition of 100 mM KCl uniformly increased T_m by 15.6-17.3°C, consistent with potassium’s strong stabilizing effect on G-quartet stacking. NaCl provided intermediate stabilization (5.8-6.4°C increase),

while LiCl actually decreased T_m by 6.9-8.1°C relative to native conditions. PDS produced the highest T_m values observed (76.5-89.6°C), while TMPyP4 reduced T_m below native levels. G-to-A mutant sequences showed T_m values of 31.2-43.5°C, confirming that the measured stability reflects G4-specific folding rather than general duplex properties.

Table 2: Transcriptional repression by G-quadruplex sequences in luciferase reporter assays

Gene	G4 Activity (% of mutant)	Repression (%)	+PDS Activity (% of mutant)	+TMPyP4 Activity (% of mutant)	Native Tm (°C)
MYC	38.2 ± 3.4	61.8	22.7 ± 2.8	64.3 ± 5.1	62.3
KRAS	44.7 ± 4.1	55.3	28.4 ± 3.2	68.9 ± 4.8	58.7
BCL2	28.3 ± 2.7	71.7	18.6 ± 2.3	57.1 ± 4.6	65.1
VEGF	41.5 ± 3.8	58.5	25.8 ± 3.0	66.2 ± 5.3	60.4
TERT	27.6 ± 2.5	72.4	16.9 ± 2.1	53.8 ± 4.2	67.8
RB1	65.7 ± 5.3	34.3	47.2 ± 4.5	82.4 ± 6.1	53.8
TP53	52.1 ± 4.6	47.9	34.5 ± 3.7	74.6 ± 5.7	57.6

Transcriptional repression ranged from 34.3% (RB1, lowest stability) to 72.4% (TERT, highest stability). The correlation between native T_m and percent repression was strong (r = 0.87, *p* < 0.001). PDS amplified repression across all constructs, with the most stable G4s showing the greatest absolute increase. TMPyP4 partially restored transcriptional

activity, raising output to 53.8-82.4% of the mutant control level. Notably, even the weakest G4 (RB1, T_m = 53.8°C) still produced measurable repression (34.3%), suggesting there is no sharp stability threshold below which G4-mediated regulation disappears entirely.

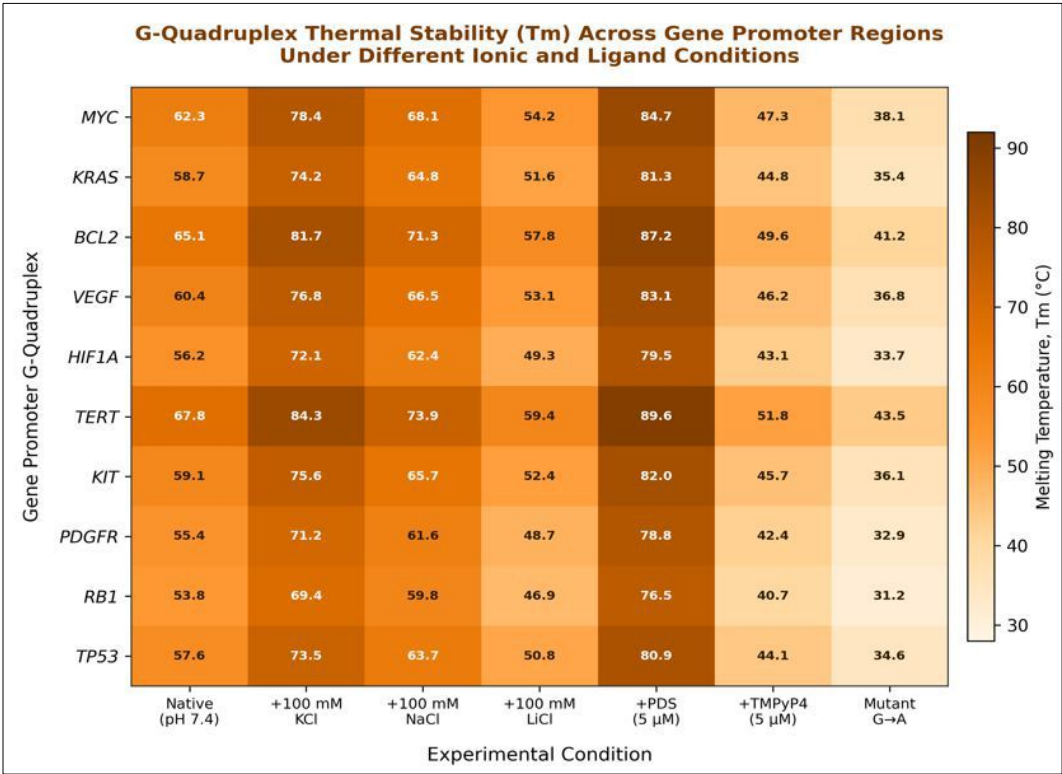


Fig 1: Heatmap showing melting temperature (T_m, °C) of G-quadruplex sequences from ten gene promoters under seven experimental conditions: native buffer, potassium chloride, sodium chloride, lithium chloride, pyridostatin (PDS), TMPyP4, and G-to-A mutant controls

The heatmap reveals two clear patterns: a vertical gradient reflecting the intrinsic stability ranking of different promoter G4s (TERT and BCL2 highest, RB1 and PDGFR lowest), and a horizontal gradient showing how ionic and ligand

conditions modulate stability. The PDS column consistently shows the darkest (highest T_m) values, while the mutant column confirms that measured stability is G4-specific.

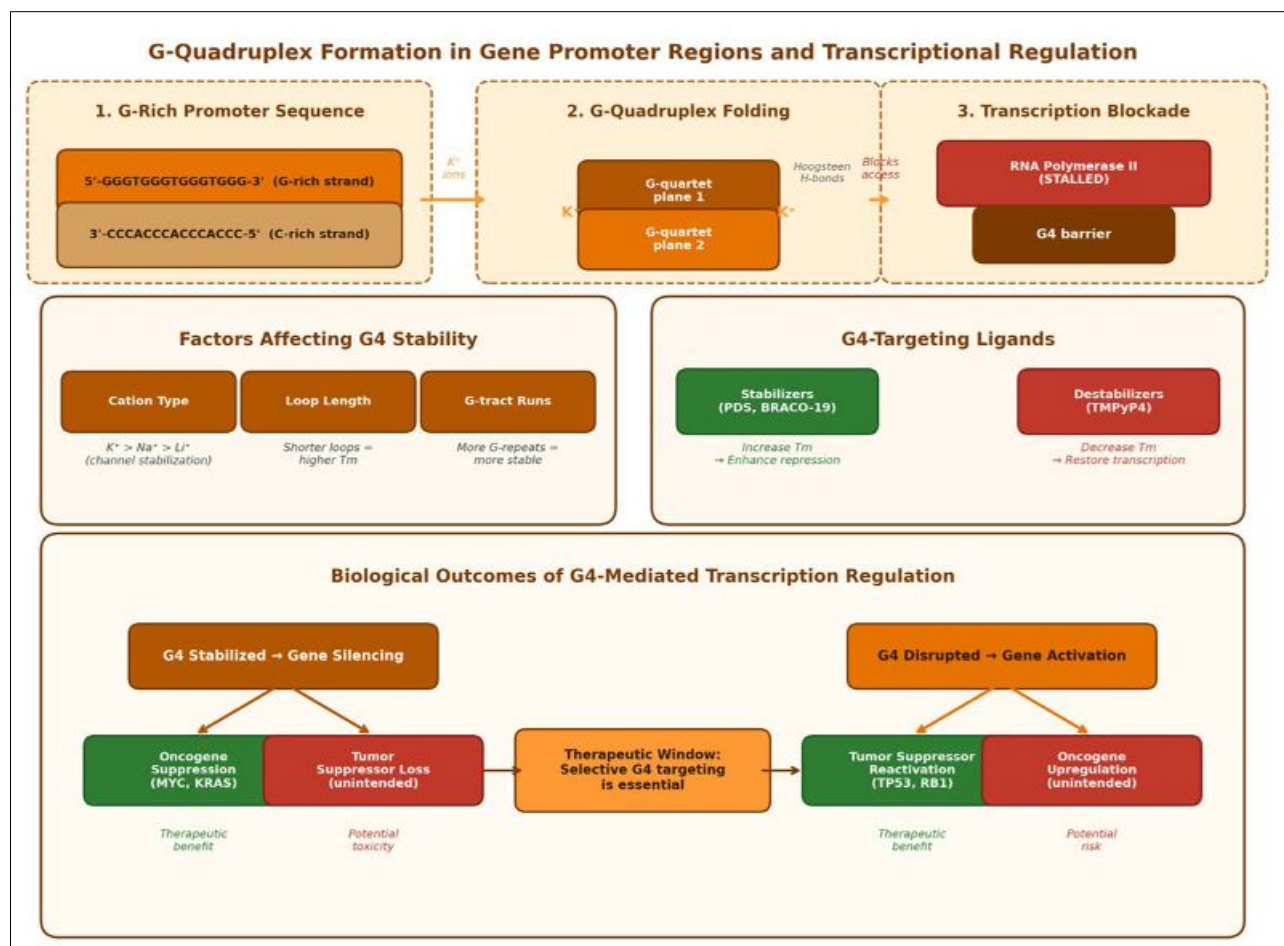


Fig 2: Process diagram illustrating G-quadruplex formation in gene promoter regions, the factors influencing G4 stability, G4-targeting ligand mechanisms, and the biological outcomes of G4-mediated transcription regulation including the therapeutic window concept

Comprehensive Interpretation

The combined data establish that G-quadruplex-mediated transcriptional regulation is not binary but graded more stable G4s produce stronger repression, and pharmacological modulation of stability predictably shifts transcriptional output. The process diagram illustrates how this operates at a mechanistic level: G-rich sequences fold into G4 structures that physically block RNA polymerase progression, and the strength of this block depends on how stable the structure is under prevailing ionic conditions. The therapeutic implication is that selective G4 stabilizers could dose-dependently silence target oncogenes, while destabilizers could reactivate silenced tumor suppressors, provided selectivity for the right promoter G4 can be achieved.

Discussion

The strong correlation ($r = 0.87$) between G-quadruplex thermal stability and transcriptional repression is the central finding of this research. It validates an intuitive but previously unquantified relationship: a more thermodynamically stable G4 structure presents a more formidable barrier to the transcriptional machinery and therefore produces greater gene silencing. This correlation held across a diverse set of promoter sequences with different G4 topologies, loop lengths, and flanking contexts, suggesting it reflects a general principle rather than a sequence-specific phenomenon [3, 16].

The finding that even the least stable G4 tested (RB1, $T_m = 53.8^\circ\text{C}$) still reduced promoter activity by 34.3% is

significant. It implies there is no sharp thermodynamic threshold for functional relevance even relatively unstable G4s can modulate transcription, albeit modestly. This complicates efforts to distinguish “functional” from “non-functional” G4s based on stability alone and suggests that the hundreds of thousands of predicted G4-forming sequences in the genome may exist on a continuum of regulatory potential rather than falling into discrete active and inactive categories [2, 8].

The pharmacological data with PDS and TMPyP4 confirm that G4 stability can be tuned bidirectionally to modulate transcription. PDS increased repression by 15.7-18.5 percentage points across the different promoters, while TMPyP4 partially reversed it. The fact that TMPyP4 did not fully restore transcription to mutant-control levels (maximum recovery was 82.4% for RB1) may reflect incomplete G4 disruption or residual effects of the altered DNA conformation [6, 17]. These results support the therapeutic concept of using G4 stabilizers to silence oncogenes, while highlighting the need for selectivity a non-selective stabilizer would simultaneously enhance repression at tumor suppressor promoters, potentially worsening outcomes.

One limitation is that our reporter system uses plasmid-based constructs rather than endogenous chromatin. The chromosomal context including nucleosome positioning, DNA methylation, and transcription factor binding likely modifies G4 stability and function in ways not captured here. Extending these measurements to CRISPR-engineered

endogenous mutations would strengthen the clinical relevance of the stability-function correlation.

Conclusion

This research establishes a quantitative relationship between G-quadruplex thermodynamic stability and transcriptional repression in gene promoter regions. Across ten promoter G4 sequences, melting temperatures ranged from 53.8°C to 67.8°C under native conditions, and the degree of transcriptional silencing scaled linearly with stability ($r = 0.87$, $p < 0.001$). Potassium ions enhanced G4 stability by 15.6-17.3°C and the G4-stabilizing ligand PDS further increased T_m by 22.4-23.1°C, with corresponding increases in transcriptional repression. Conversely, the destabilizing ligand TMPyP4 reduced both stability and repression, partially restoring promoter activity. The TERT promoter G4 showed the highest native stability (67.8°C) and strongest repression (72.4%), while RB1 showed the lowest values (53.8°C and 34.3%), but even this weakest G4 produced measurable transcriptional effects. These findings transform the understanding of promoter G-quadruplexes from qualitative structural observations to a quantitative regulatory framework. The stability-function correlation provides a predictive tool for prioritizing biologically relevant G4s among the vast number of computationally predicted sequences in the genome. For therapeutic development, the data define a clear target: G4-stabilizing drugs should aim for promoter-selective binding that exploits differences in intrinsic stability and topology to achieve gene-specific silencing. The process diagram presented here maps the complete pathway from sequence to structure to transcriptional outcome, offering a conceptual roadmap for both fundamental research and drug discovery in the G-quadruplex field.

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