ANALYSIS OF THE EFFECTS OF BCL-2 PROMOTER G-QUADRUPLEX FORMATION ON PROTEIN EXPRESSION

By

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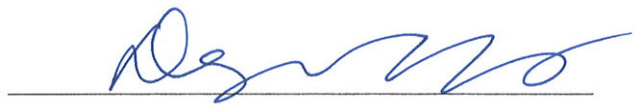

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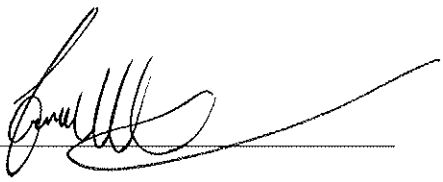
Signed: 

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Abstract

Bcl-2 is an anti-apoptotic protein that has been implicated in a number of human diseases, including some cancers. Within the P1 promoter region, which is involved in 80-90% of bcl-2 transcriptional control, a G-rich region (Pu39) can form various monomeric G-quadruplex folding structures, each with four of the six runs of multiple guanines present. The midG4 and 5'5G4 structures have been found to be the most stable and are the potential targets of comparative studies on transcriptional effects. Plasmids containing a luciferase reporter gene under the control of this bcl-2 P1 promoter region, along with mutants to alter or eliminate G-quadruplex formation, were constructed in order to study the ability of Pu39 to control transcription. Initial luciferase assay results in HeLa and HEK293 cells suggest that mutants designed to isolate certain G-quadruplex structures caused an overall decrease in protein expression while mutants designed to knock out major folding structures caused an increase in protein expression. DNA mutant constructs in conjunction with quadruplex-specific, quadruplex-interacting drugs appear to consistently decrease protein activity, supporting the idea that the G-quadruplex has an inhibitory effect on transcription.

1 Introduction

Apoptosis, or programmed cell death, is a crucial process for the human body to retain homeostasis. It allows for the replacement of older or sicker cells and acts as a guard against cancer by disposing of cells that have left the normal replication cycle. When this natural process is disturbed, the body will react accordingly. If too much apoptosis is occurring, the area of interest can atrophy. If too little apoptosis is occurring, cells can continue to exist and grow without check, often leading to tumor formation.

1.1 B-cell Lymphoma 2

B-cell lymphoma 2, commonly referred to as bcl-2, is the founding protein of the bcl-2 protein family and is naturally found in many mammals. The entire bcl-2 family is comprised of apoptosis regulatory proteins, with bcl-2 falling under the anti-apoptotic category. The idea that an excess of anti-apoptotic regulators can contribute to cancers is now a widely accepted cancer mechanism. While bcl-2 excess may not necessarily cause cancer directly, when interacting with other proteins, such as the proto-oncogene c-Myc, bcl-2's effects become more pronounced (1). One earlier hypothesis suggested that members of the bcl-2 family would regulate apoptosis via interactions through inhibition of the apoptosis protease activating factor 1, or apaf-1 (2). However, two later studies would conclude that bcl-2 did not significantly interact with apaf-1, instead, the BH4 domain of the protein would interact with voltage-dependent anion channels, VDACs, in order to inhibit passage of cytocrome c, a protein associated with the apoptotic process (3, 4). More recently, it has also been shown to down-regulate apoptosis via interaction with the mitochondrial apoptosis-induced channel, MAC (5). In mitochondrial regulation of apoptosis, bcl-2 specifically prevents the formation of the MAC, preventing the release of cytochrome c and the apoptotic cascade.

Regardless of mechanism, bcl-2 remains a protein of interest to many cancer researchers for its observed inhibitory role in apoptosis. More precisely, we are interested in how the bcl-2 protein may contribute to cancer formation and persistence. It has been observed that an excess of the bcl-2 protein may contribute to the resilience of certain cancers by preventing the apoptotic response. This correlation has been found in both prostate cancer and lymphocytic leukemia (6, 7). However, despite these findings, much of the mechanisms behind the regulation of the bcl-2 protein production are largely unexplored. This study attempts to identify the role the bcl-2 promoter region has on overall transcription efficiencies with the intent that, by better understanding how bcl-2 production may be up-regulated, advances may be made on ways to lower protein production in tumor cells. In particular, we are looking at a DNA secondary structure, known as the G-quadruplex, whose formation may control transcription.

1.2 G-quadruplex

1.2.1 G-quadruplex Formation

The G-quadruplex is a DNA secondary structure that forms through the interactions of guanine-rich DNA sequences. Four guanines can interact through Hoogsteen hydrogen bonding to form a guanine tetrad. Most commonly from current research, it has been shown that either two or three of these tetrads will stack, resulting in what is known as a G-quadruplex (Figure 1A) (8). In theory, a quadruplex can only form from single-stranded DNA, limiting its existence to telomeric sequences and in single-stranded DNA regions. Single-stranded DNA, which we are studying, comes from helicase activity creating an area of negative supercoiling in the DNA during transcription, opening up a single-stranded region and, thus, potentially allowing for quadruplex formation.

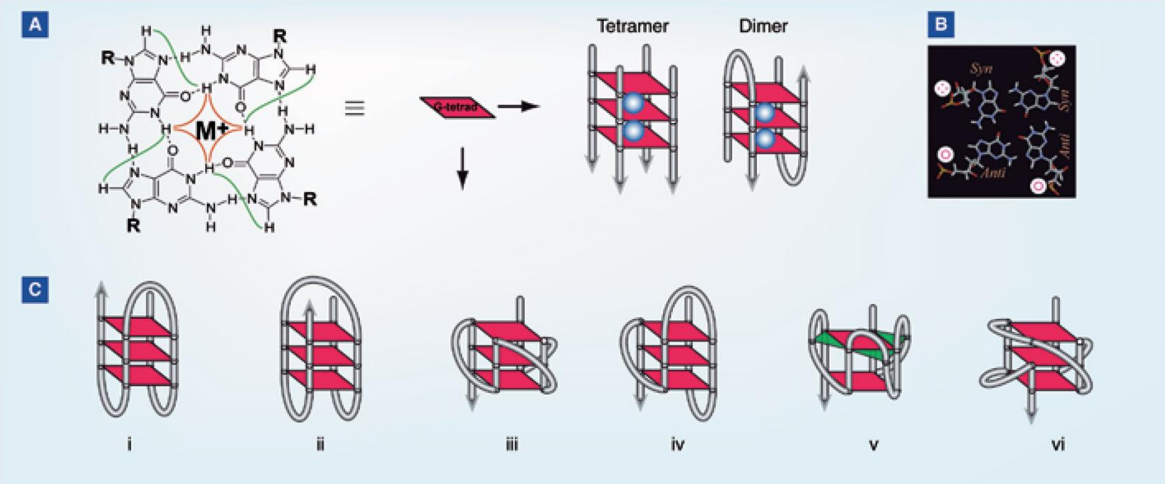

Figure 1. (A) G-tetrad and the formation of tetrameric and dimeric quadruplexes. (B) The *syn* and *anti* forms of guanine. (C) Monomeric G-quadruplex folding structures (Image courtesy of Yang & Okamoto, 2010 (8)).

Physiological conditions identified in the study of G-quadruplexes have shown the importance of various ion concentrations, specifically that of sodium and potassium. These ions, in the form of salts, can sit within the channel created by the tetrads, allowing for the stabilization of the structure. Though intercellular salt concentration fluctuates around 100 mM, the potassium ion, K+, has been see to be the most stabilizing in sustaining quadruplex structures, with other ions such as sodium being less effective, though still usable (8, 9). Varying salt concentrations are now frequently used in research to promote quadruplex formation.

G-quadruplex formation has been extensively explored through the use of nuclear magnetic resonance (NMR) and X-ray crystallography. From this, it is currently understood that, while G-quadruplex formation (especially for parallel-stranded structures) tends to follow a sequence pattern in which guanine runs are separated by one or two other bases, several structures have been known to contain loops that are ten or more bases long. Additionally, the guanines in each tetrad can adopt either a *syn* or *anti* glycosidic conformation, which can change the direction of the DNA. While these conformations are easier to determine in dimer or tetramer quadruplexes, monomeric quadruplexes are much more complicated in terms of structure. As a result of directional and loop length variations, there are many structures a guanine-rich, single-stranded region can form (Figure 1) (8). A G-quadruplex may fold in any conformation based on the sequence of the G-rich region and stability of the structure formed.

1.2.2 Drug Development

From the developing understanding of the G-quadruple structure, many small molecule drug compounds have been developed in an attempt to stabilize the G-quadruplex. Many of these involve one of two categories of interactions with binding molecules: groove binding and stacking interactions. Groove binders bind in the curved side grooves caused by the natural

DNA structure (10). Drugs that have stacking interactions can bind to either the top or bottom of a quadruplex formation (11). For this study, two well-known stacking drug compounds were used: TMPyP2 and TMPyP4 (Figure 2). These two structural isomers differ on the outside of the porphyrin ring, with TMPyP2 having less affinity towards the G-quadruplex. At the same time, it should be noted that porphyrin ring compounds, classified as symmetrical fused ring structures, may not bind the quadruplex as specifically as other compounds, such as the crescent-shaped quindoline (11). In spite of their reduced specificity, TMPyP2 and TMPyP4 still act as probes for quadruplex formation. By using TMPyP2 as a standard, we were able to observe the effects of the stronger stabilizer, TMPyP4, on the quadruplex within our bcl-2 P1 promoter region.

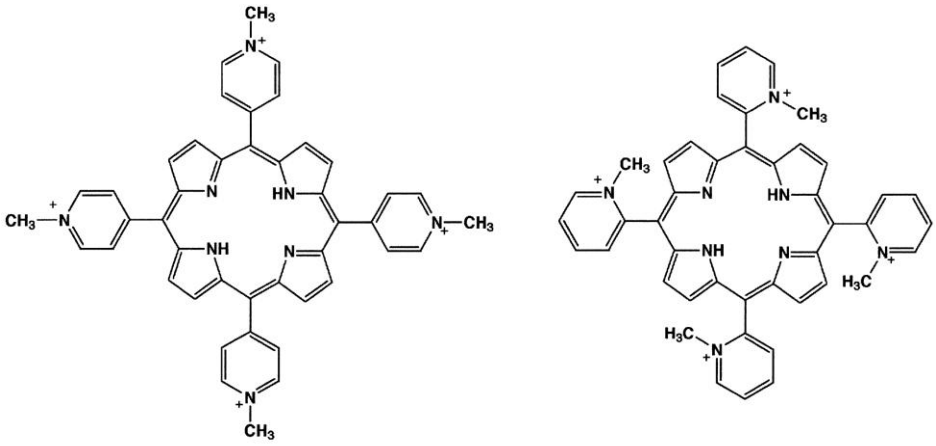

Figure 2. Molecular structures of TMPyP4 and TMPyP2.

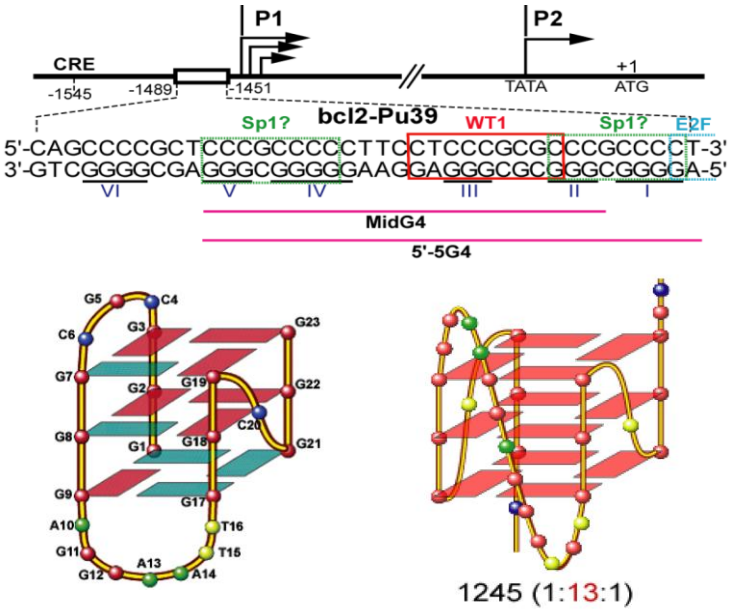

Figure 3. Major folding structures of the Pu39 region in the bcl-2 promoter. MidG4 (left) (13a) and 5'5G4 (right) (5'5G4 structure created in Yang lab), as well as the guanine runs involved in each structure (top)

1.2.3 Formation in Bcl-2

Prior research has indicated that the G-quadruplex is present in the promoters of many protein sequences and often involved in gene regulation (12). This study revolves around the hypothesis that the Pu39 quadruplex-forming region, a 39 bp G-rich element (-1490 to -1452 relative to the translational start site) in the bcl-2 P1 promoter sequence, acts as a critical control element for bcl-2 transcription. Earlier NMR studies have shown that the midG4 and 5'5G4 folding structures are the most stable quadruplexes of the various isoforms within the Pu39 region. The midG4 structure utilizes the middle four runs of the G-rich sequence, while the 5'5G4 structure uses runs 1, 2, 4, and 5 of the G-rich sequence (Figure 3) (13a, b). Due to the stability of these two major folding structures, studies can be performed to determine differences in their effects on transcription. This study focuses on understanding how the G-quadruplex is formed in this particular promoter region, the contribution of each structure to the overall transcriptional activity of this region, and how to stabilize and destabilize the structure using small molecule compounds which could be developed as therapeutic drugs.

2 Materials and Methods

2.1 Site-directed Mutagenesis

Polymerase Chain Reaction (PCR) is commonly used for site-directed mutagenesis. Using the Quikchange II XL mutagenesis kit from Stratagene and later the Phusion High-Fidelity PCR Kit from Thermo Scientific for its GC-rich PCR capabilities, various point mutations were made in the bcl-2 promoter-containing pGL3-Basic (Promega) vector to determine the contributions of various guanine runs to promoter activity. This pGL3-bcl2 wild-type plasmid was created using the bcl-2 promoter insert (-1287 to -1640 bp upstream from the translational start site) from Linda Boxer's lab in the plasmid LB334, accessed through Addgene (14). The

bcl-2 promoter acts on the luciferase gene from pGL3-Basic to alter luciferase translation. Combining the promoter region with pGL3-Basic allows for the promoter to control the luciferase gene. This plasmid also contains ampicillin resistance to control for incomplete mutations. Oligonucleotides used as primers for the PCR were obtained from Sigma-Aldrich.

Primer design was done with reference to the Quikchance II XL Mutagenesis Kit protocol. Several key points should be kept in mind when designing primers for site-directed mutagenesis. Length should include mutations and 10-15 bases in both 5' and 3' directions, preferably keeping the total length between 25 and 45 bases (15). Additionally, GC content should be kept above 40% and primer melting temperature (T_m) should be higher than 78 ℃. However, at the same time, it was found that high GC content could hinder PCR efficiencies (16) Therefore, primer length was sometimes extended in order to lower GC content of the primers, though never beyond 30 bases total.

When running the PCR, cycling conditions were kept fairly stable with the exception of the annealing temperature. Depending on the kit and protocol used, denaturation was done at 95-98 ℃ for 30-60 seconds and elongation/extension at 68-72 ℃ for 30-60 seconds (15, 17). For annealing temperatures, the initial temperature for all experiments started at 55℃ and was adjusted accordingly to troubleshoot and obtain the ideal annealing temperature which would produce the most accurate mutations. The annealing temperatures used in the course of this study ranged from 53 to 64℃. Due to the G-rich nature of the Pu39 region, DMSO was used with the Phusion High-Fidelity PCR kit in order to lower the melting temperatures of the oligonucleotides anywhere between 0.5 and 0.6℃ per 1% DMSO used (17). Again, depending on the protocol used, the number of cycles for heating and cooling ranged from 20 to 30 cycles. The list of sequences obtained through this method is included in Figure 4.

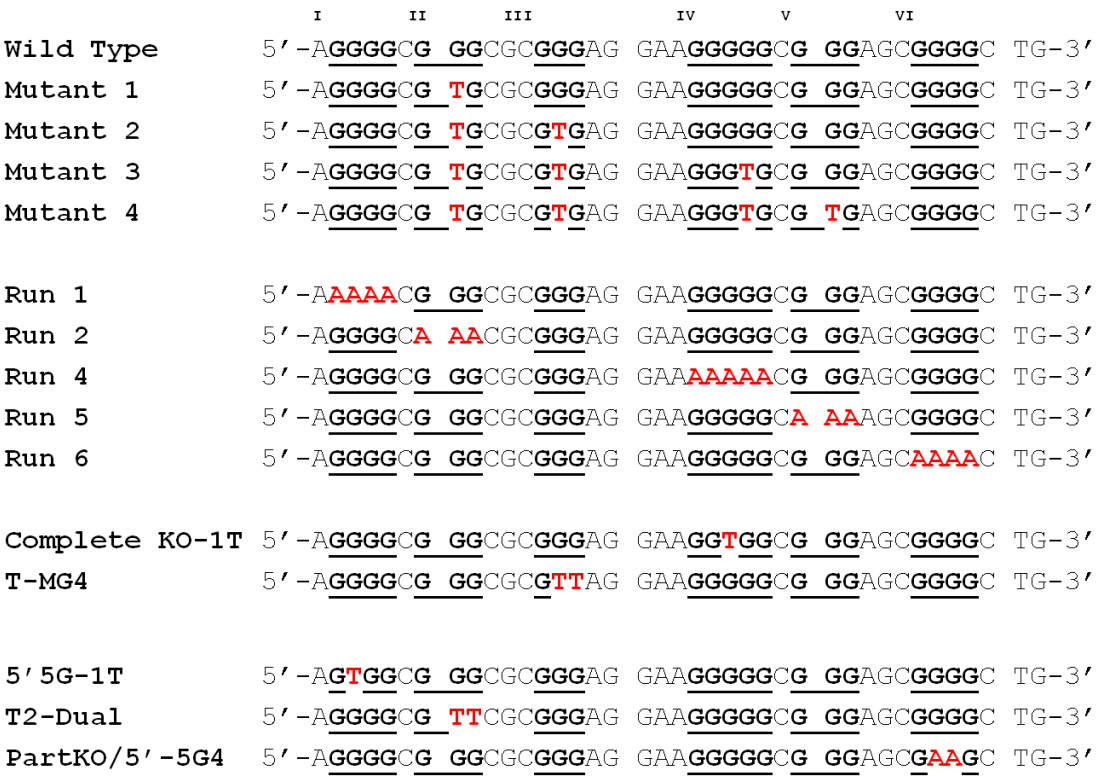

Figure 4. Mutants of the Pu39 sequence obtained through site-directed mutagenesis. Mutations marked in red.

Once the initial plasmid mutants were constructed using PCR, the PCR products were digested with DpnI in order to remove all wild-type methylated DNA, leaving the mutated DNA created from the enzymatic reactions. These mutated plasmids were then transformed into DH5-α E. coli cells for DNA amplification and cultured on agar plates. Both media and agar plates used in this amplification were made with the Lennox lysogeny broth recipes, which includes 10g/L of NaCl, as well as containing 50 µg/mL of ampicillin in the media and 100 µg/mL in the agar plates to prevent unwanted bacterial growth. After amplification and culture, the DNA was purified using either the QIAprep Spin Miniprep Kit or a QIAfilter Plasmid Midiprep Kit from Qiagen. Mutants were verified using agarose gel electrophoresis for size and DNA sequencing for accuracy.

2.2 Cell Culture

HEK293 (normal human embryonic kidney) and HeLa (cervical cancer) cell lines were cultivated in DMEM and RPMI from Invitrogen, respectively. All media contained 10% fetal bovine serum and 100 mg/mL of both penicillin and streptomycin.

2.3 Transfection and Luciferase Assay

Cells were incubated in multi-well culture plates 24 hours prior to transfection. Depending on well size, the number of cells used ranged from 80,000 cells/well for 24-well plates to 500,000 cells/well for 6-well plates. Mutants were transfected with FugeneHD at a ratio of 3:2 μL Fugene:μg DNA into cells at about 80% confluency. For transfections in the absence of drug, a Renilla luciferase plasmid, pRLTK, was cotransfected for normalization at a ratio of about 100 times lower than the luciferase containing plasmid in order to avoid interference with luciferase expression. In the presence of drug treatments, compounds were added concurrently with the transfection mixtures. Cells were lysed in the wells 24 hours after

treatment using Passive Lysis Buffer from Promega. The luminescence assay was performed using Promega's Dual-Luciferase Reporter Assay Kit. Each transfection and assay pair was run in triplicate to allow for discrepancies in transfection efficiency and cell count.

3 Results

3.1 Baseline Protein Expression Levels in Mutants

To obtain a standard with which to compare to, the mutations were cotransfected with pRLTK for normalization. Incubation time was set for 24 hours and the experiment was performed with both HeLa and HEK293 cells (Figure 5). The pGL3-bcl2 wild-type vector contained the luciferase protein sequence downstream of the promoter insert, allowing the promoter to activate luciferase production. Through this, it was possible to analyze promoter function through this reporter gene. Luciferase assays allowed for the differences in protein production to be expressed through quantitative means.

The initial mutations, Mutants 1 through 4, were created to progressively restrict the middle tetrad in the midG4 folding structure. We had expected to see some kind of trend as we mutated more guanine runs. However, in both HeLa and HEK293 cell lines, Mutant 2 seemed to have a higher protein expression than the other three mutants, suggesting that there was something different about the quadruplex in that mutant. Mutant pGL3_CKO was designed to knock out any potential G-quadruplex formations by restricting Run 4. The slight increase in protein in both cell lines with this mutant was thought to reflect some inhibiting qualities of the two major G-quadruplex structures. Mutant MidG4 attempted to remove Run 3, inhibiting the midG4 folding structure. One highly replicable point observed was that this mutant would consistently lower protein production to below that of the empty pGL3Basic vector. Full run mutations, Runs 1, 2, 4, 5, and 6, were designed in order to test the importance of each individual

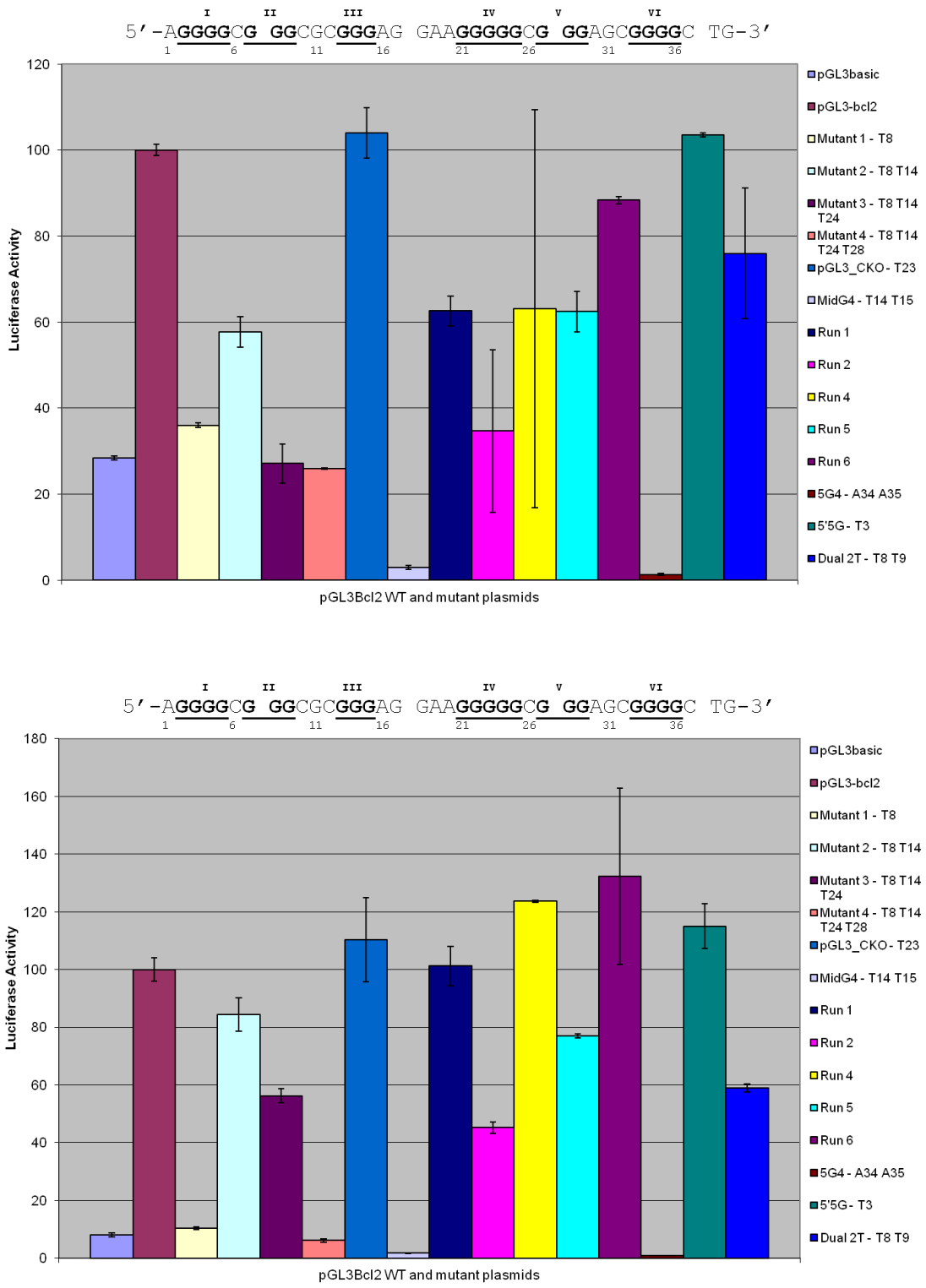

Figure 5. Luciferase assays of mutants in HeLa (top) and HEK293(bottom). Values were compared to pGL3-bcl2, which was set to 100%.

guanine run. However, the results differed widely between the two cell lines, giving us inconclusive data. Conflicting data was obtained between Run 6 and mutant 5G4, which would have isolated the major structures in a manner similar to Run 6. The two mutants differed in only two guanines within run 6 and yet yielded extreme differences in protein production. Mutant 5'5G was thought to inhibit the 5'5G folding structure and demonstrate any regulating effects of the other major folding structure, midG4. While Mutant 5'5G showed slight inhibitory qualities in HeLa cells, it had more of an activating effect in HEK293. Finally, mutant Dual2T was created to knock out both major folding structures. Both cell lines showed a decrease in protein expression with this mutant construct.

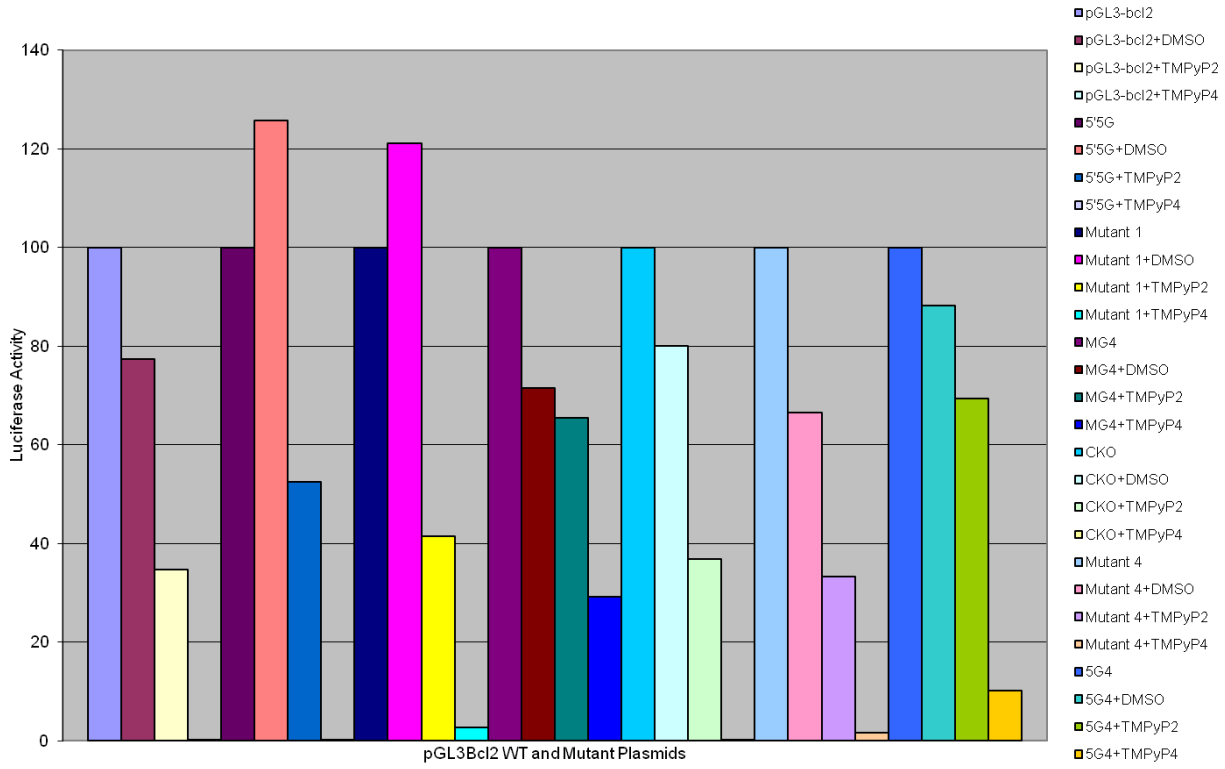

Figure 6. Luciferase assays of each mutant with addition of TMPyP2 and TMPyP4 in HeLa cells.

3.2 Drug Testing with TMPyP2/TMPyP4

Transfections with quadruplex-targeting drugs TMPyP2 and TMPyP4 were performed to test the potential presence of quadruplex formation, and also to observe the definitive effects on the G-quadruplex. In this experiment, HeLa cells were transfected with the mutants, left to incubate for a short amount of time, usually around 30 minutes, and finally dosed with 2.5 μM of either TMPyP2 or TMPyP4 resuspended in DMSO. After 24 hours of incubation with the drugs, the cells were harvested in the same manner as the prior experiments with luciferase. In this experiment, full run mutations Run 1, 2, 4, 5, and 6 were not used under the suspicion that the mutant stocks needed to be remade. The firefly luciferase assay process gave the corresponding protein levels, with each mutant set containing a nontreatment group, a DMSO treatment, and the two drug treatments (Figure 6). The results were normalized via protein quantitation within each mutational set, allowing for comparison between treated and untreated cells. From this experiment we feel that we have identified the potential effects of the G-quadruplex within the bcl-2 P1 promoter sequence. This Pu39 region seems to function as an inhibitor of protein production.

4. Discussion

In this study, we observed the complexities existing within the 370-bp bcl-2 P1 promoter insert present in our plasmid system. While previous NMR studies on this system have revealed the midG4 folding structure to be a major contributor to G-quadruplex formation, there are at least two others, a 5'G4, and even a 3'G4, that may also form. It appears that, in attempting to knock out specific guanine runs through point mutations, we likely caused other folding patterns to occur. This resulted in the consistent appearance of a G-quadruplex, as whenever quadruplex

interacting drugs were introduced to the system, the protein expression levels would consistently drop.

While it would appear that the Pu39 region acts to inhibit protein production through the formation of a G-quadruplex, our initial data lacks definitive proof to support this claim. For instance, the mutants that should have removed any major folding structures, Mutant 4 and pGL3_CKO, had vastly differing levels of protein expression. If we were to take into account the idea that the quadruplex acts as an inhibitor, we would expect to see more of an increase in protein expression in both mutants. A further inspection of this Pu39 region reveals numerous binding proteins and transcription factors, such as SP1 and WT1 (18, 19). Most notably, the consistent results from mutant MidG4 may be attributed to WT1 binding. It is possible that a specific mutation is somehow allowing some inhibitory transcription factor to bind better. Another consideration is that quadruplex formation could also better facilitate transcription factor binding. A separate project from this lab is currently looking at whether or not the WT1 protein binds to the quadruplex. Therefore, we have been working towards a new set of mutants which would avoid tampering with these protein binding sites as well as investigating the effects of transcription factors in the complete absence of quadruplex.

We must also take into account the possibility that these mutations did not act as they were intended to. Most importantly is the observation that the two quadruplex-interacting drugs acted similarly on all mutants tested. This suggests either of two possibilities, the first being that the mutations did not do what they were designed to do, and that quadruplexes were still forming within the region despite efforts to design mutants that would inhibit all formation. However, there is only weak evidence to support this because, as stated previously, these two drug compounds may not be entirely specific for certain folding structures. In order to design drug

compounds that target specific structures, we would first need to fully understand how the Pu39 region behaves. The other consideration is that there are other G-quadruplexes which can form within our promoter insert outside the Pu39 region. This phenomenon would prevent accurate determination of the effects of the Pu39 reading while giving the results obtained from the drug compound experiments.

In conclusion, this Pu39 region of the bcl-2 P1 promoter is extremely complex, both in terms of potential quadruplex structures and its natural environment. With further experimental design, we have currently created several restriction sites in the LB334 insert, with minimal sequence changes, in hopes that it will aid in mutagenesis and cloning. These restriction sites will also allow for the removal of specific regions, even allowing for the retention of transcription factor binding sites while removing the bases involved in quadruplex formation. There needs to be a more specific analysis of this region and its components before any concrete conclusions can be formed about functions of this G-quadruplex bcl-2 promoter region.

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