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The *RAD17* promoter sequence contains a potential tail-dependent G-quadruplex that downregulates gene expression upon oxidative modification

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Abstract

Our laboratory has recently proposed that the oxidation of guanine (G) to 8-oxo-7,8-dihydroguanine (OG) in G-rich promoter regions of DNA repair genes can serve as a regulatory mechanism of gene transcription. These regions also have the potential to fold into G-quadruplexes (G4). The human *RAD17* promoter sequence has such a region in the template strand of the gene. In this work, the potential G-quadruplex sequence (PQS) of the *RAD17* gene promoter was interrogated in different sequence contexts. With two extra nucleotides of the native sequence on either side of the G4, the structure was found to fold into a hybrid-like G4, similar to the hybrid-1 fold that the human telomere sequence can adopt. With only one nucleotide on either side of the PQS, the topology of the structure was observed to be mixed, and without extra nucleotides on the ends, the sequence adopted a parallel fold. Next, the sequence was studied with synthetic incorporation of the oxidative modification OG into specific sites and installed into the promoter of plasmids with a luciferase gene. These plasmids were transfected into a human cell line to observe the effect of the G4s on transcription. The *RAD17*PQS was found to decrease luciferase expression with the presence of OG that is consistent with *RAD17* expression under oxidative stress. This serves as an example of how oxidative modification could affect transcription in the context of a G4.

INTRODUCTION

Biomolecular chemical modifications resulting from oxidation by reactive oxygen species (ROS) have been implicated in the initiation and progression of aging, coronary disease, neurological diseases, and cancer.¹ These disease states represent the deleterious effects of cellular ROS; on the other hand, ROS, such as H₂O₂, are also cellular signaling agents that oxidize specific proteins as part of the regulatory network for cells to respond to increasing ROS levels.² All biomolecular components are susceptible to oxidation via ROS. In DNA, the electron-rich guanine (G) heterocycle is most susceptible to oxidation to yield many

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Supporting Information

Copies of ¹H-NMR spectra, CD spectra, ThT fluorescence, and T_m values for all *RAD17* 5' Track, G-to-T mutated sequences, and for sequences bearing 8-BrG and OG

Notes

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oxidatively modified products, in which 8-oxo-7,8-dihydroguanine (OG) is a key product identified in vivo.³ Further, a positive correlation between ROS and OG levels is well documented.⁴ Thus, our laboratory and others have proposed oxidation of G to OG in promoter regions of DNA can serve as a signaling agent to regulate gene transcription.⁵⁻⁷ This phenomenon of gene activation via G oxidation in a promoter region has been documented in the *VEGF*,⁸ *SIRT1*,⁹ *TNF- α* ,¹⁰ and *BCL2*¹¹ genes in mammals. Under what circumstances and how globally does G oxidation in gene promoters modulate gene transcription are questions we have asked.

Our work on the topic of gene regulation by G oxidation to OG in a promoter region initially focused on the *VEGF* promoter because it contains a G-rich region in the coding (i.e., non-transcribed) strand that is responsible for gene regulation, and this region is prone to oxidation.¹² More interestingly, the G-rich region is a potential G-quadruplex sequence (PQS) that can adopt a parallel-stranded G-quadruplex (G4) fold,¹³ and this feature has been proposed as part of the regulatory mechanism.¹⁴ Thus, we undertook studies to understand if G oxidation to OG in the *VEGF*PQS could activate transcription via favorable G4 formation.¹⁵ Briefly, the ability to synthesize OG site-specifically into the *VEGF*PQS for regulation of a luciferase reporter gene transfected into mammalian cells allowed the following observations to be made. When OG is present in the *VEGF*PQS in the coding strand, the reporter gene was turned on via a mechanism that involved base excision repair (BER) to remove the OG and yield a helix destabilizing abasic site (AP) that provided the thermodynamic drive to shift the structure to the G4 state. The *VEGF*PQS possesses five G tracks that allow flexibility in the structure to shift the damaged G-run into a large loop and stabilize the G4 structure with incorporation of the undamaged 5th G track.¹⁶ This new structure provides a platform for protein recruitment and gene activation. We followed this study with another that placed the *VEGF* promoter PQS into the template strand of the luciferase reporter gene.¹⁷ In the second study, we found transcription was turned off by the presence of OG in the G-rich context in the template (transcribed) strand. The work suggested OG and the G4 fold work together to block transcription preinitiation leading to the decrease in gene expression observed. In conclusion, G oxidation to OG in the G4 context in the coding strand of a gene promoter can turn transcription on, and in contrast, the same chemistry and sequence in the template strand of a gene promoter turns transcription off. These observations led us to question whether mechanisms like these could occur in other genes for transcriptional regulation.¹⁸

Thus, we conducted a bioinformatic analysis to identify hundreds of PQSs in human DNA repair gene promoters.¹⁸ We conducted initial structural studies on 30 of these sequences to establish G4 folding, and five were taken into the reporter gene studies to demonstrate the PQSs responded to G4 ligands by altering the transcription levels of a luciferase gene in glioblastoma cells. During the study, a PQS was found in the template strand of the promoter for the *RAD17* gene (Figure 1A) that furnished a very interesting and unique circular dichroism (CD) spectrum for a promoter G4 (Figure 1B). The spectrum was nearly identical to that observed for the hybrid folds of the human telomere sequence,¹⁹ this observation was fascinating because nearly all of the promoter G4s characterized adopt parallel or mixed folds and rarely hybrid folds.²⁰ Thus, we elected to further interrogate the *RAD17*PQS and the results are presented herein. Previous cellular studies have identified the *RAD17*PQS to

be possibly folded in the cellular context,^{21,22} and oxidative stress conditions result in down regulation of the gene.²³ These two cellular observations suggest oxidation of G to OG in the *RAD17PQS* in the template strand of the gene promoter may function to down regulate transcription. Herein, the structural results support the conclusion that this sequence can adopt a hybrid G4, as long as tails of two nucleotides each exist on the 5' and 3' ends of the sequence. When the tails were removed, the sequence was found to adopt a parallel-stranded G4. This observation provides a cautionary note to the G4 community that inclusion of the natural sequences flanking a promoter G4 may dramatically alter the fold observed. Lastly, studies were conducted to demonstrate that when an oxidation prone G in this sequence is modified to OG, the gene is down regulated, on the basis of a luciferase reporter assay in human glioblastoma cells.

METHODS

Oligodeoxynucleotide Preparation

The oligodeoxynucleotides (ODNs) were synthesized by the DNA-Peptide Core Facility at the University of Utah using commercially available phosphoramidites. The ODNs were cleaved and deprotected following standard protocols and then purified using an anion-exchange semi-preparative HPLC column with a linear gradient from 25–100 %B over 30 min while monitoring the UV/vis absorbance at 260 nm. (A = 10% CH₃CN/90% ddH₂O, B = 1 M LiCl, 25 mM Tris in 10% CH₃CN/90% ddH₂O, pH 8, flow rate = 3 mL/min.) The purification salts were removed by dialysis against ddH₂O for 36 h at 4 °C. The dialyzed DNA was lyophilized to dryness and resuspended in ddH₂O followed by measurement of the absorbance at 260 nm and using the primary sequence to estimate the extinction coefficient to determine the DNA concentration. The DNA was then annealed at the specified concentration for each experiment via heating to 95 °C for 5 min and then slowly cooling to room temperature over 3 h followed by storage at 4 °C for 24 h prior to analysis.

NMR Studies

The ¹H-NMR experiments were conducted on an 800-MHz NMR spectrometer running the Watergate solvent suppression pulse sequence. The spectra were recorded at a DNA concentration of 300 μM in 20 mM KP_i (pH 7) with 50 mM KCl at 24 °C.

Circular Dichroism, T_m, and Thioflavin T Fluorescence Studies

The circular dichroism (CD) spectra were recorded using a 1-mm path length quartz cuvette on samples with a DNA concentration of 10 μM in a buffer containing 20 mM lithium cacodylate, 140 mM KCl, and 12 mM NaCl (pH 7, 20 °C) over a range of 200–320 nm. Thermal denaturation experiments were conducted to obtain T_m values on samples with a DNA concentration of 2 μM in the same buffer conditions by heating the samples at 1 °C/min and monitoring the absorbance changes at 295 nm. The thioflavin T fluorescence studies were measured on samples containing 1 μM DNA and 0.5 μM fluorophore, and the λ_{em} was scanned from 440–700 nm with a λ_{ex} = 425 nm. The λ_{em} = 490 nm was monitored for each sequence studied.

Mapping Sites of Oxidation via Piperidine Cleavage

The annealed strands were 5' radiolabeled and oxidized with the one-electron oxidant $\text{CO}_3^{\bullet-}$ following literature protocols.²⁴ The sites of oxidation were revealed via treatment with freshly prepared 1 M piperidine at 90 °C for 2 h. This method was previously demonstrated to cleave most G oxidation products in a quantitative yield.²⁴ After cleavage, the samples were lyophilized to dryness and resuspended in loading dye and then electrophoresed on a 20% denaturing PAGE at 45 W for 2 h. The sites of oxidation were visualized by storage phosphor autoradiography.

Plasmid Preparation

The plasmids were constructed with the psiCHECK2 plasmid (Promega) that contains the Renilla luciferase (Rluc) and firefly luciferase (luc) genes. Restriction-free cloning was utilized to insert the *RAD17* promoter G4 sequences, followed by transformation of these plasmids to competent *E. coli*. The plasmids were isolated from these cells by a miniprep kit (Qiagen). Site-specific modification of OG was synthesized in short oligonucleotides and inserted into the plasmid via literature methods.¹⁵

Cell Culture Studies

Glioblastoma cells (U87-MG) were purchased from ATCC. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS, 20 $\mu\text{g/mL}$ gentamicin, 1 $\times$ glutamax, and 1 $\times$ nonessential amino acids. Transfection experiments were conducted in white, 96-well plates with X-tremeGene HP DNA transfection agent (Roche) with 200–750 ng of plasmid following literature protocols.¹⁵ The Dual-Glo luciferase assay (Promega) was conducted following the manufacturer's protocol to monitor Rluc and luc expression levels. Each experiment was conducted in four replicates as recommended by the manufacturer. The measured luminescence values were converted into normalized relative response ratios (RRR) that is the luminescence of Rluc divided by the luminescence of luc (i.e., $\text{RRR} = \text{Rluc/luc}$).

RESULTS AND DISCUSSION

Characterization of the *RAD17* Promoter G-Quadruplex

The *RAD17* promoter PQS was initially studied with four G tracks with two nucleotides of the natural sequence as tails on both the 5' and 3' ends (*RAD174* Track, Table 1). Studies with the human telomere sequence found that omission of these natural sequence overhangs can impact the structures of the G4 fold.²⁵ In the first set of studies, upon purification, the samples were annealed in NMR buffer (20 mM KPi pH 7.0, 50 mM KCl, 22 °C) at a concentration of 300 μM DNA to maximize signals in ^1H -NMR studies. The diagnostic imino protons of G:G Hoogsteen base pairs (10–12 ppm)^{26,27} in G-tetrads of G4 structures were identified to support folding of the sequence to a G4 (Figure 1C). There exist 12 major peaks in this region of the spectrum consistent with one predominant G4 fold in solution. This observation was highly unusual compared to ^1H -NMR spectra for other PQSs in promoter regions of various DNA repair genes that showed an array of broad signals in the imino proton region.¹⁸

The DNA sample was then diluted to be analyzed by other spectroscopic methods. The CD spectrum of the *RADI7PQS* was recorded in a buffer to mimic the K^+ and Na^+ human intracellular concentrations (20 mM lithium cacodylate pH 7.0, 140 mM KCl, 12 mM NaCl) at 10 μ M DNA concentration. The spectrum showed a $\lambda_{\max} \approx 292$ nm, $\lambda_{\text{shoulder}} \approx 264$ nm and $\lambda_{\min} \approx 245$ nm that is nearly identical to the one observed for a hybrid fold which the human telomere sequence can adopt (Figure 1B).¹⁹ The T_m value (Figure 1D) for the sequence was measured by observing the decrease of absorbance at 295 nm with increasing temperature from 20 °C to 95 °C. The T_m value was found to be 69.5 ± 1.0 °C, higher than physiological temperature, and thus this sequence has the potential to fold in cellulo.²⁸ In an additional experiment, the fluorescence emission enhancement (Figure S1) of the G4-specific fluorophore thioflavin T (ThT) was measured in the presence of the *RADI7PQS* following a literature protocol that found enhancement of fluorescence of ThT of >20 $Fl_{490\text{nm}}/Fl_o$ in the presence of a folded G4.²⁹ With a value of 18.9 ± 0.3 $Fl_{490\text{nm}}/Fl_o$, the folded sequence produced results inconsistent with G4 formation; however, recent studies have identified ThT binding is more favorable with parallel-stranded G4s than hybrid-like G4s as the *RADI7* sequence appears to adopt.³⁰

The sequence with five G domains (*RADI75 Track*) also had signals in the imino proton range of the $^1\text{H-NMR}$ spectrum (Figure S2). However, there were more peaks than observed for the *RADI74 Track* sequence. The peaks were also less defined, suggesting there exist many possible structures in solution, consistent with having five runs of Gs with one run of Gs having five nucleotides (Figure 1A).¹⁶ The CD spectrum for the *RADI75 track* (Figure S2) also had signals at $\lambda \approx 290$ nm, $\lambda \approx 263$ nm, and a $\lambda_{\min} \approx 245$ nm. However, the signal at $\lambda \approx 263$ nm was larger than that of $\lambda \approx 290$ nm that is opposite of the spectrum for the four track sequence; the wavelengths at which $\lambda_{\max}$ and $\lambda_{\text{shoulder}}$ are found in this spectrum are switched compared to the four track sequence. This may indicate a different folding pattern or a mixture of folding topologies when compared to literature spectra.³¹ The T_m value was found to be 68.6 ± 0.8 °C (Figure 1D), similar to the value that was obtained for the four-track sequence. The sequence also gave a fluorescence enhancement value of 63.3 ± 4 $Fl_{490\text{nm}}/Fl_o$ (Figure S1). With these observations, the *RADI75 Track* sequence can fold at physiological conditions and is possibly more polymorphic with incorporation of the fifth run of Gs compared to the four-track sequence.

The sequence was then analyzed to elucidate the possible structure into which the quadruplex folds. The PQS has three runs of three Gs that could all participate in folding, and one run of four Gs of which only three Gs would participate in folding at a given time (Figure 1A). This would mean that one of the four Gs in the last run would be part of a loop. To find out which G this would be, two new sequences (*RADI7T18* and *RADI7T21*) were studied (Table 1) with separate G-to-T substitutions that would prevent that nucleotide from participating in the formation of the G-tetrad core. Comparison of the new sequence and the wild-type sequence would help identify which G would be best for the folding of the G4.³² These DNA samples were obtained, purified, and prepared as above. In the $^1\text{H-NMR}$ spectra (Figure S3), the *RADI7T18* showed defined peaks at the 10–12 ppm range while the *RADI7T21* showed peaks that were less defined. The CD spectrum (Figure S4) for T18 was similar to the one for the wild-type *RADI7* sequence with a $\lambda_{\max} \approx 292$ nm, $\lambda_{\text{shoulder}} \approx 264$ nm and $\lambda_{\min} \approx 245$ nm that is indicative of a hybrid-like fold. The *RADI7T21*

sequence, however, had a $\lambda_{\max} \approx 266$ nm and $\lambda_{\min} \approx 241$ nm that is consistent with a parallel-stranded G4 on the basis of a comparison to literature sources.³³⁻³⁶ The T_m values for the *RAD17*T18 and T21 sequences were 61.6 ± 1.4 °C and 73.0 ± 0.5 °C (Figure 1D), respectively, thus both sequences have the potential to fold in cellulo. The fluorescence enhancement assay with ThT found values of 26.0 ± 1.7 Fl_{490nm}/Fl_o for *RAD17*T18 and 74.2 ± 2.9 Fl_{490nm}/Fl_o for *RAD17*T21 (Figure S1), thus they have the capability to fold into G4s. Considering that the data for the sequence *RAD17*T18 matched the data collected for the original *RAD174* Track sequence, the G that was substituted to a T in that sequence (at position 18) does not participate in the core of the G4, but instead occupies a loop position in these contexts.

Using the *RAD17*T18 sequence (Figure 2A) to decrease variability in folding of the G4, a study was then performed to support a structural model of the hybrid G4 fold. A key characteristic of hybrid G4s is that they possess both *syn* and *anti* G nucleotides. To probe which Gs were *syn* and which were *anti*, separate sequences (Table S1) were obtained with individual substitutions at each of the guanines to 8-bromoguanine (8-BrG). Because 8-BrG is known to strongly favor the *syn* conformation, its substitution for a G either stabilizes or destabilizes the structure depending on the preferred fold,³⁷⁻⁴⁰ i.e. the *syn* conformation is confirmed if the structure is stabilized and the opposite assignment is made (*anti*) if 8-BrG substitution is destabilizing. The sequences that gave CD spectra (Figure S5) similar to that of the one obtained for the *RAD17*T18 and wild-type sequences were considered to have the substituted G in the *syn* conformation. The spectra that gave different signatures were considered to have the substituted G in the *anti* conformation. Assignments were further determined with T_m studies in which a lower melting temperature would correlate with lower stability, and thus the position was identified as being in the *anti* orientation (Figure 2B). With these data, the folding pattern of the sequence (Figure 2C) was found to be similar to that of the hybrid 1 structure that the human telomere sequence can adopt.^{19, 41,42}

Effect of Tails on the *RAD17* Promoter G-Quadruplex

To learn more about the structure that the *RAD17*PQS adopts, the absence and presence of tails was studied to find their effects on folding. Several sequences were interrogated, each varying in the number of nucleotides in the tails (Table 1). The molecularities of the different sequences were interrogated via size-exclusion chromatography (Figure S6) in which the results support intramolecular folding for the sequences under the conditions studied. With five nucleotides of the natural sequence on both the 5' and 3' ends (*RAD175* nt), there were a few defined peaks in the 10–12 ppm region of the ¹H-NMR spectrum, and the sequence adopted a hybrid-like fold based on CD spectroscopy (Figure 3A and 3B). The T_m was 64.9 ± 0.2 °C and fluorescence enhancement value with ThT of 21.6 ± 1 Fl_{490nm}/Fl_o (Figures 1D and S1). The data collected for the *RAD175* nt sequence concur with the original sequence and suggest that it folds into a hybrid G4; thus, adding more nucleotides at the ends of the sequence in addition to the two original nucleotides as single-stranded tails does not change the folding pattern.

Because adding nucleotides does not change the folding pattern, the next sequences studied had a reduced number of nucleotides in the tails. With one-nucleotide tails on both ends

(*RAD17* 1 nt), there were still some defined peaks in the ^1H -NMR spectrum (Figure 3A), and the sequence adopted a similar fold to the wild-type *RAD17* sequence. However, the signal at $\lambda \approx 292$ nm decreased while the signal at $\lambda \approx 264$ nm increased (Figure 3B). This may indicate that a fully parallel fold may be in equilibrium with a hybrid fold. The T_m value was measured to be 64.7 ± 0.5 °C, and the sequence showed a fluorescence enhancement value of $12.2 \pm 0.3 \text{ Fl}_{490\text{nm}}/\text{Fl}_o$ (Figures 1D and S1) with thioflavin T. The fluorescence enhancement was lower than expected for a G4, but considering there was an enhancement observed, there is still the possibility that the sequence can fold to a parallel or anti-parallel structure.

With the interesting observation that mixed topologies could be formed with one nucleotide on both ends of the G4 forming sequence, more studies were performed on additional sequences to elucidate whether a single nucleotide on one end could give similar results. First, a sequence with one nucleotide at the 5' end (*RAD17* 5' Tail) was studied. For the CD spectrum (Figure S7), this sequence gave a $\lambda_{\text{max}} \approx 263$ nm and $\lambda_{\text{min}} \approx 241$ nm, which was similar to the spectrum of the *RAD17*T21 sequence. Thus, the *RAD17* 5' Tail sequence adopts only parallel folds, on the basis of the CD spectrum. The T_m value was found to be 71.0 ± 0.4 °C (Figure 1D), thus the G4 has the possibility of folding in physiological temperatures. As for the sequence with one nucleotide at the 3' end (*RAD17* 3' Tail), the CD spectrum was similar to that obtained for the *RAD17* 5' Tail sequence (Figure S7) with a $\lambda_{\text{max}} \approx 262$ nm and $\lambda_{\text{min}} \approx 240$ nm. The T_m was found to be 68.6 ± 0.9 °C (Figure 1D). Thus, this sequence can adopt a parallel-stranded G4 with one nucleotide at either the 5' or 3' end of the sequence.

A final sequence without tails (*RAD17*Tailless) was studied. Without nucleotides flanking the G4-forming sequence, there was a signal as a broad hump of undefined peaks between 10–12 ppm in the ^1H -NMR spectrum (Figure 3A). The CD spectrum (Figure 3B) gave a $\lambda_{\text{max}} \approx 262$ nm and $\lambda_{\text{min}} \approx 240$ nm that indicates a parallel fold. The T_m value was found to be 76.6 ± 0.2 °C (Figure 1D), similar to the melting temperatures that have been obtained for the other sequences. The sequence also had a fluorescence enhancement value of $88.1 \pm 7.1 \text{ Fl}_{490\text{nm}}/\text{Fl}_o$ (Figure S1). The results were expected considering that with one nucleotide on either end of the G4, the sequence adopted parallel folds. Without any nucleotides on either end, the sequence adopts a parallel fold.

Based on the results of the tail studies, we surmise that the tails have a major effect on the G4 folding pattern the PQSs can adopt. A change in tail length correlating with a change in folding of a G4 was previously observed for the human telomere sequence where a different number of nucleotides in overhanging tails would result in different hybrid folds.²⁵

However, *RAD17* is unique in a subtle change in the tail length can change the structure from a hybrid to a parallel-folded G4. This brings a cautionary note to those studying G4 structure in synthetic oligodeoxynucleotides; sequence truncation can potentially lead to erroneous interpretations.

Oxidation of the *RAD17* promoter PQS

In addition to modifying the tail lengths of the *RAD17* promoter PQS, studies to determine the impact that oxidation could have on the behavior of the sequence were conducted. The

RAD174 Track sequence was exposed to the one-electron oxidant $\text{CO}_3^{\bullet-}$ and subsequent analysis via gel electrophoresis revealed hotspots for oxidation at G nucleotides at the 5' face of the G runs as well as Gs in the loops. The data are consistent with previous studies finding similar hotspots of oxidation for G4 folds.¹⁶ A common G oxidation product from this reaction is 8-oxo-7,8-dihydroguanine (OG);^{3,43,44} therefore, two sequence variations with oxidative modifications (Table 1) at one of these hotspots were selected to study: one with oxidation at the 5'-terminus (position 13) of a G run, and one with oxidation in a loop position (position 18). First, oxidation was site-specifically incorporated in the *RAD17PQS* as OG in place of the guanine. After synthesis of the sequence containing OG, the DNA was purified and annealed in the same buffer previously described for collecting the CD spectra.

Initially, the CD spectra and T_m values (Figure S8) were obtained for the sequences with five domains. With oxidation at position 13 (core), there was a $\lambda_{\text{max}} \approx 290$ nm, $\lambda_{\text{shoulder}} \approx 262$ nm and $\lambda_{\text{min}} \approx 237$ nm. Compared to the wild-type sequence, the signal at 290 nm increased, which may indicate a more hybrid-like structure in solution. On the other hand, the oxidation at position 18 (loop) gave a spectrum very similar to that of the five track sequence without damage with a $\lambda_{\text{max}} \approx 264$ nm, $\lambda_{\text{shoulder}} \approx 290$ nm and $\lambda_{\text{min}} \approx 238$ nm. The CD spectra suggest that with the OG in a core position of the sequence, the folding is less polymorphic and a more defined fold is formed. Comparing the spectrum of position 13 to that of the four track sequence without damage, we found they were nearly identical. By introducing the damage at that position, the structure may adopt the same fold as the four-track sequence. Meanwhile, damage in a loop position (such as with position 18) does not appear to affect the folding pattern as the CD spectrum is similar to that of the five-track sequence without damage. These results concur with previous results found by our laboratory for the fifth track of the *VEGFPQS* in which we described how the presence of OG in a core position can cause a rearrangement of the G4 structure to include the fifth track instead of the track with the modification.¹⁶ The T_m values obtained for the sequences were >60 °C verifying that both sequences can fold into quadruplexes at physiological temperatures.

With the four-track sequence, modification at position 13 showed a CD spectrum (Figure S8) with $\lambda_{\text{max}} \approx 288$ nm, $\lambda_{\text{shoulder}} \approx 261$ nm and $\lambda_{\text{min}} \approx 238$ nm. The modification at position 18 gave a similar spectrum (Figure S8) with a $\lambda_{\text{max}} \approx 290$ nm, $\lambda_{\text{shoulder}} \approx 266$ nm and $\lambda_{\text{min}} \approx 242$ nm. Both of these spectra are similar to the one obtained for the wild-type four-track sequence without modification, indicating that OG at either position does not affect the folding pattern. However, the T_m values tell a different story. Both values are above physiological temperatures, but with an oxidative modification in the sequence, the values decreased compared to the original sequence. At position 13, the presence of OG decreased the T_m by almost 30 °C, while at position 18, the T_m decreased by about 6 °C (Figure S8). The large decrease in melting temperature at position 13 indicates that the presence of OG in the core position greatly destabilizes the G-quadruplex as the OG will not participate in the Hoogsteen base pairing of the tetrad.⁴⁵ The small decrease at position 18 indicates that the damage in the loop position only marginally affects the fold considering the damage will not participate in the fold of the G4.

***RAD17* Promoter PQS in a Human Cell Line**

In the final set of studies, some of the G4-forming *RAD17* sequences (Table 1) were inserted into the SV40 promoter sequence of the Renilla luciferase gene in a reporter plasmid to understand whether the folding of the G4 in human cells could have an effect on gene expression. The sequences were incorporated into the plasmid following a method that was previously reported by our laboratory.¹⁵ The firefly luciferase gene that was also present in the plasmid was not modified and was therefore used as an internal standard. The modified plasmids were then transfected into human glioblastoma cells (U87-MG). The luciferase expression levels were obtained and normalized to obtain relative response ratios (RRRs).

The *RAD175* Track sequence was inserted into the plasmid, and to study the effect of oxidation on expression levels, *RAD175* OG13 or *RAD175* OG18-containing plasmids were similarly prepared. Compared to the normal wild-type (all guanine) G4 sequence, both positions of oxidative modification decreased expression of the Renilla luciferase gene (Figure 4A). Similar results were found for the *RAD174* Track PQS when comparisons were made to sequences with OG at positions 13 and 18. Both positions decreased expression of the Renilla luciferase gene (Figure 4B) in the U87-MG cells. Our laboratory previously found that with the presence of a G-quadruplex containing OG or AP modifications in the template strand of a gene, such as with the *RAD17*PQS that is native in this strand, there was also a downregulation of expression of the gene.¹⁷ This concurred with previous reports that oxidation downregulates the expression of the *RAD17* gene in U937 human lymphoma cancer cells.²³ The magnitude of the downregulation is somewhat smaller with *RAD17* construct compared to the previously studied *VEGF* promoter, possibly due to the less stable hybrid G4 fold of *RAD17* compared to the parallel G4 of *VEGF*.¹⁷

CONCLUSIONS

In this report, we characterized the PQS that resides in the promoter region of the human *RAD17* gene. The sequence was found to have the ability to fold to a G4 consistent with a hybrid-like topology under model physiological conditions. This was found to be different from other PQSs found in promoter regions that usually adopt parallel folds.²⁰ Different nucleotide lengths of tails were also found to alter dramatically the folding pattern from hybrid-like to parallel topologies. With at least two nucleotides of the native sequence flanking both sides of the PQS, the G4 remained a hybrid-like fold. However, when less than two nucleotides were present on either the 5' or 3' sides, the folding of the G4 shifted to a parallel topology. The study with tail length serves as a cautionary note for any experiments performed on PQSs, including structural characterization by NMR, especially if conclusions are to be made based on the folding pattern of the G4 in physiological conditions.

Folding of the *RAD17* sequence was also examined when either of two oxidation-prone Gs were synthetically substituted by OG, the common oxidation product of G. When OG was introduced into a core position (13), the G4 structure was hybrid-like and not very dynamic, while OG in a loop position (18) led to a less well-defined fold. Importantly, both points of modification still led to stably folded G-quadruplexes.

The *RAD17* promoter PQS was then interrogated in human cells with or without the oxidatively modified base OG. The sequences were inserted into plasmids, transfected into human glioblastoma cells, and a luciferase assay was performed. The assay revealed that the sequences containing OG decreased the expression of luciferase compared to undamaged sequences, concurring with the cellular observation that in the presence of oxidative stress, the expression levels of *RAD17* decreased.²³ This work is an example of how a G-quadruplex in a promoter region of a gene coupled with the oxidative modification OG affects transcription, and this modification could possibly assist in regulation of gene expression via initiation of DNA repair.¹⁷

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Depiction of the location of the *RAD17*PQS in the promoter region of the gene. (B) The CD spectrum obtained for the sequence with four domains of G runs. (C) Imino region (10–12 ppm) of the ¹H NMR spectrum of the sequence. (D) T_m values obtained for sequences studied.

Figure 2.

Studies with 8-BrG substitution of Gs to elucidate folding pattern of *RAD17* promoter PQS.

Red represents a G in the *syn* position, blue represents a G in the *anti* position. (A) T18

Sequence used for studies with positions of the Gs marked. (B) T_m data obtained of

sequences with 8-BrG substituted in different positions. (C) Depiction of the fold that the

parent sequence adopts.

Figure 4.

Expression levels of Renilla luciferase relative to firefly luciferase (RRR) measured for (A) the *RAD17* 5 Track sequence with and without oxidative modifications, and (B) the *RAD17* 4 Track sequence with and without oxidative modifications. Significance values for each comparison of data were calculated via a Student's *t* test. Significance at * $P < 0.05$ or ** $P < 0.01$ is indicated.

Table 1.

The *RAD17* promoter PQSs studied. A red X indicates a substitution from a G to an OG.

Name	Sequence
<i>RAD174</i> Track	5' – CC GGG A GGG ACT GGG CT GGGG CA – 3'
<i>RAD175</i> Track	5' – CC GGG A GGG ACT GGG CT GGGG CAG GCT GGGGG CG – 3'
<i>RAD17T18</i>	5' – CC GGG A GGG ACT GGG CT TGGG CA – 3'
<i>RAD17T21</i>	5' – CC GGG A GGG ACT GGG CT GGGT CA – 3'
<i>RAD175nt</i> Tails	5' – CGGCC GGG A GGG ACT GGG CT GGGG CAGGC – 3'
<i>RAD171nt</i> Tails	5' – C GGG A GGG ACT GGG CT GGGG C – 3'
<i>RAD175'</i> Tail	5' – C GGG A GGG ACT GGG CT GGGG – 3'
<i>RAD173'</i> Tail	5' – GGG A GGG ACT GGG CT GGGG C – 3'
<i>RAD17</i> Tailless	5' – GGG A GGG ACT GGG CT GGGG – 3'
<i>RAD175</i> OG13	5' – CC GGG A GGG ACT XGG CT GGGG CAGGCT GGGGG CG – 3'
<i>RAD175</i> OG18	5' – CC GGG A GGG ACT GGG CT XGGG CAGGCT GGGGG CG – 3'
<i>RAD174</i> OG13	5' – CC GGG A GGG ACT XGG CT TGGG CA – 3'
<i>RAD174</i> OG18	5' – CC GGG A GGG ACT GGG CT XGGT CA – 3'