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Site-Specific Probing of Oxidative Reactivity and Telomerase Function Using 7,8-Dihydro-8-oxoguanine in Telomeric DNA

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Abstract: Telomeres at the ends of human chromosomes contain the repeating sequence 5′-d[(TTAGGG)$_n$]-3′. Oxidative damage of guanine in DNAs that contain telomeric and nontelomeric sequence generates 7,8-dihydro-8-oxoguanine (8OG) preferentially in the telomeric segment, because GGG sequences are more reactive in duplex DNA. We have developed a general strategy for probing site-specific oxidation reactivity in diverse biological structures through substitution of minimally modified building blocks that are more reactive than the parent residue, but preserve the parent structure. In this study, 8OG was substituted for guanine at G_8, G_9, G_{14}, or G_{15} in the human telomeric oligonucleotide 5′-d[AGGGTTAG$_8$G$_9$GTT AG$_{14}$G$_{15}$-GTTAGGGTGT]-3′. Replacement of G by 8OG in telomeric DNA can affect the formation of intramolecular G quadruplexes, depending on the position of substitution. When 8OG was incorporated in the 5′-position of a GGG triplet, G quadruplex formation was observed; however, substitution of 8OG in the middle of a GGG triplet produced multiple structures. A clear correspondence between structure and reactivity was observed when oligonucleotides containing 8OG in the 5′-position of a GGG triplet were prepared in the quadruplex or duplex forms and interrogated by mediated electrocatalytic oxidation with $Os(bpy)_3^{2+}$ (bpy = 2,2′-bipyridine). The rate constant for one-electron oxidation of a single 8OG in the 5′-position of a GGG triplet was $(6.2 \pm 1.7) \times 10^4$ M^{-1} s^{-1} in the G quadruplex form. The rate constant was 2-fold lower for the same telomeric sequence in the duplex form ($(3.0 \pm 1.3) \times 10^4$ M^{-1} s^{-1}). The position of 8OG in the GGG triplet affects telomerase activity and synthesis of telomeric repeat products. Telomerase activity was decreased significantly when 8OG was substituted in the 5′-position of the GGG triplet, but not when 8OG was substituted in the middle of the triplet. Thus, biological oxidation of G to 8OG in telomeres has the potential to modulate telomerase activity. Further, small molecules that inhibit telomerase by stabilizing telomeric G quadruplexes may not be as effective under oxidative stress.

The ends of mammalian chromosomes contain long tracts of repeating guanine-rich sequence. In humans, the telomeric repeat is 5′-d[(TTAGGG)$_n$]-3′ and persists for up to 10 kilobase pairs.[1] Most of the telomeric sequence is double-stranded; however, a portion of the G-rich strand extends past the duplex as a single-stranded overhang of variable length that depends on the organism.[2] In vitro electron microscopy of telomeres from mammals[3] and *Trypanosoma brucei*[4] has shown that telomeres end in a large loop formed by invasion of the single-stranded overhang into the duplex. Base-pairing of the single-stranded G-rich sequence likely protects the ends of linear chromosomes from erroneously being recognized as double-stranded DNA breaks by DNA repair enzymes.[5,6]

Single-stranded DNAs that contain guanine-rich sequences, such as the telomeric repeat sequence, fold to form a structural element known as a G quadruplex.[7] G quadruplexes can assemble through parallel alignment of four separate DNA strands, parallel or antiparallel association of two hairpins, or folding of a single strand (Figure 1).[8] In general, G quadruplexes form preferentially in the presence of K^+ or Na^+,[8] but quadruplex formation has been observed also with Cs^+[9] or NH_4^+.[8] Formation of G quadruplexes is accelerated by cationic free-base porphyrins or a cationic derivative of perylene.[10−17]

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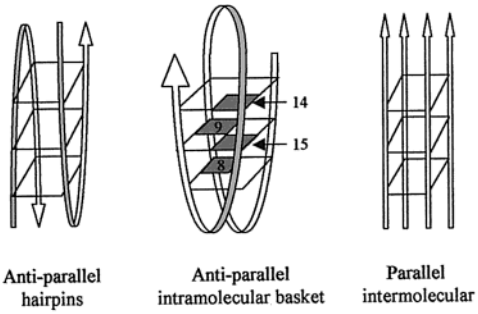

Figure 1. Possible G quadruplex structures. The nucleic acid strands are shown as arrows; the 3′-end is the head of the arrow. The large unfilled squares are the G quadruplex planes; smaller filled squares represent individual guanines. The numbers correspond to the positions of the guanosines that were individually replaced by 7,8-dihydro-8-oxoguanosine in this study. Guanosines 9 and 14 are anti while 8 and 15 are syn.

Interest in telomeric guanine quadruplexes is due to their potential role in regulating telomerase, an enzyme that regenerates truncated telomeric DNA.[9,16,18,19] In normal somatic cells, telomerase is expressed only at low levels, and shortened telomeres arrest cell division because chromosomal fidelity cannot be preserved.[20,21] Cancerous cells, however, express telomerase at levels higher than normal cells[22] because shortened telomeres block unrestricted cell growth.[23] Elevated expression of telomerase is a hallmark of cancer cells, and much effort is being expended currently to inhibit telomerase.[12−17,24−35]

In general, telomerase inhibitors act either by interfering with telomerase directly or by altering the structure of the DNA substrate, thereby disrupting telomerase activity. One strategy involves molecules that induce telomeres to fold into guanine quadruplexes, inhibiting telomerase binding.[12−17,24,32−35] Another method uses chain-terminating agents such as modified nucleotides and reverse transcriptase inhibitors that stop extension of telomeric DNA.[29−31,36] Anti-sense oligonucleotides composed of modified RNA or peptidic nucleic acids (PNA) also inhibit telomerase, presumably by binding to telomeric ends or to the RNA template of telomerase.[25,26] Small molecules that recognize telomeric sequences and the RNA/DNA duplex formed by telomerase/telomere association are effective telomerase inhibitors.[28] Facilitation of G quadruplex assembly as a means to inhibit telomerase may combine with a decrease in telomere length induced by damage to improve the effectiveness of such small molecule inhibitors. The mode of telomerase inhibition for the natural product telomestatin has not been reported, although the compound does not inhibit reverse transcriptases other than telomerase.[27]

To our knowledge, no studies on telomerase inhibition have been published in which active telomeric DNA was chemically modified at a specific position to produce an inactive substrate. Blackburn et al. have shown that mutations in telomeric DNA cause end-to-end fusions of chromosomes[37] and that mutations in telomerase RNAs produce changes in telomerase activity.[38,39] In addition, 6-thioguanine[40] and inosine[41−43] have been substituted into quadruplex-forming DNAs to probe quadruplex structure, but telomerase activity was not investigated. Recent work by Oikawa et al. indicates that in vitro oxidation of a mixed nontelomeric−telomeric double-stranded oligonucleotide forms 7,8-dihydro-8-oxoguanine (8OG) preferentially in the telomeric segment of the DNA.[44,45] Depending on the method of oxidation (UVA irradiation in the presence of riboflavin[45] or Cu(II) with hydrogen peroxide[44]), the site of oxidative damage is either the 5′-G or the middle position of telomeric GGG triplets. These results raise the possibility that guanine triplets in telomeric sequences act as sinks for oxidative damage in vivo,[44,45] although direct evidence − including information on site-specificity and products − is not yet available. Other lesions formed upon oxidation of guanine in telomeric sequences have not yet been identified. Base-labile lesions are produced either from initial guanine oxidation or from subsequent oxidation of 8OG.[46−50] UVA irradiation of telomeres produces both 8OG and piperidine-labile lesions ascribed to 2,6-diamino-4-hydroxy-5-formamidopyrimidine (Fapy-G).[45]

In our laboratory, we have used mediated electrochemistry to study the one-electron oxidation of nucleic acids containing native and chemically modified nucleobases.[51−58] Electrochemi-

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cal measurements provide real-time estimates of nucleobase reactivity as a function of sequence, stacking, and DNA structure.[51−58] For example, we have shown previously that guanine triplets in G quadruplex DNA are not hole traps for oxidizing equivalents as they are in duplex DNA.[56] Similarly, we have shown that the 5′-G in GG sequences in duplex DNA reacts with a one-electron oxidation rate constant that is 12-fold higher than that found for isolated guanines in duplex DNA.[55] In these experiments, the reactivity of the 5′-G in guanine doublets was obtained by selective substitution of unreactive inosine for guanine followed by deconvolution of the contributions from each guanine.[55] Hickerson et al. have shown that when guanine was replaced with 8OG and the products of oxidation followed by denaturing gel electrophoresis, a much smaller ratio in reactivity between 5′-XG and 5′-GX sequences (X = 8OG) was observed.[49] The apparent discrepancy between electrochemistry and electrophoresis results probably arises, in part, from the fact that the strand-scission products measured by electrophoresis are formed by trapping oxidized guanine (or 8OG) after the initial one-electron oxidation event. This follow-up chemistry may be less sequence selective than the initial oxidation step, resulting in a lower estimate of reactivity overall.

Since 8OG may be found in high concentrations in telomeres, we were interested in the effect of 8OG on assembly of intramolecular G quadruplexes, the reactivity of 8OG in G quadruplex versus duplex telomeric DNA, and the effect of 8OG incorporation on telomerase activity. The broader goal was to create a general methodology for probing reactivity in diverse structural contexts using site-specific substitution. We report here that site-specific substitution of 8OG for G in the oligonucleotide 5′-d[(AGGGTT)$_3$ AGGGTGT]-3′ affects the formation of intramolecular G quadruplexes in a manner that depends on the position of substitution. Further, 8OG in the telomeric G quadruplex undergoes one-electron oxidation with a faster rate constant than 8OG in a duplex telomeric sequence. Finally, substitution of 8OG into telomeric substrates shows that the position of 8OG in the GGG triplet affects telomerase activity and synthesis of telomeric repeat products. In conjunction with the results of Oikawa et al., our results suggest that 8OG formation in telomeric sequences has the potential to modulate telomerase activity and the therapeutic effects of G quadruplex-based inhibitors of telomerase.

Materials and Methods

Reagents. $Os(bpy)_3Cl_2$ was synthesized according to literature procedures[59] and recrystallized from water/acetone. $Os(bpy)_3^{3+}$ was generated according to procedures published previously.[60] Oligonucleotides **1**−**8** were supplied by the Lineberger Cancer Center Nucleic Acid Core Facility, and concentrations were determined spectrophotometrically using a Hewlett-Packard 8452A diode-array spectrometer. Water was deionized using a MilliQ plus water purification system from Millipore (ϵ = 18 mΩ). Diethylpyrocarbonate (DEPC)-treated water was prepared by standard procedures[61] and used for all PCR reactions and manipulations. Solutions of 40% acrylamide:bis(acrylamide) in a 29:1 ratio were purchased from National Diagnostics (Atlanta, GA). Tetramethylethylenediamine (TEMED) was supplied by BioRad (Hercules, CA). Ammonium persulfate (APS), tris, and boric acid were purchased from Life Technologies. Ethylenediamine tetraacetic acid (EDTA) was from Mallinckrodt.

Electrochemistry. All oligonucleotides used for electrochemistry were gel-purified as described below to remove mercaptoethanol, which is oxidized by $Os(bpy)_3^{3+}$ and is present as an antioxidant in preparations of 8OG-containing oligonucleotides. Cyclic voltammograms were collected using a BAS 100B potentiostat. Electrochemical measurements were performed in cells that have been previously described.[62] The working electrode was tin-doped indium oxide (area $\approx$ 0.32 cm^2) from Delta Technologies, Inc. (Stillwater, MN). The Ag/AgCl reference electrode was purchased from Cypress Systems, Inc. The potential was applied via a platinum wire counter electrode. Cyclic voltammograms were collected at a scan rate of 25 mV/s. A freshly cleaned ITO electrode was used for each experiment. An initial scan containing 800 mM NaCl, 50 mM NaP_i buffer, pH 7 was subtracted from each experimental cyclic voltammogram. ITO electrodes were conditioned for at least six cycles in buffer before the background cyclic voltammogram was collected. At least three experiments were performed for each data point. Digital simulation was performed as described previously using the Bioanalytical Systems software package Digisim.[53,54,56,58,63,64]

Appropriate amounts of stock solutions of **2** or **4**, NaCl, and NaP_i were combined to give solutions with final concentrations of 50 μM **2** or **4**, 800 mM NaCl, 50 mM NaPi, pH 7 after hybridization and addition of $Os(bpy)_3^{2+}$. For the samples of duplex **2** or **4**, an aliquot of full-length Watson−Crick complement was added to give a final concentration of 50 μM. G quartet or duplex **2** or **4** were prepared by heating solutions to 90 °C for 5 min and cooling to room temperature over 2.5 h. After cooling, the appropriate amount of a stock solution of $Os(bpy)_3^{2+}$ in water was added to give a final $Os(bpy)_3^{2+}$ concentration of 25 μM.

General Radiolabeling and Polyacrylamide Gel Electrophoresis (PAGE). Oligonucleotides were piperidine-treated and gel-purified in a 20% polyacrylamide gel containing 7 M urea according to standard procedures.[61] The purified oligonucleotides were radiolabeled with [γ-^{32}P]-ATP (10 mCi/μL, New England Nuclear) using T4 polynucleotide kinase (Life Technologies) as previously described[56] or according to the Intergen (Purchase, NY) TRAPeze telomerase detection kit manual (see below). Nondenaturing PAGE was performed on a 16% polyacrylamide gel containing 100 mM KCl that was run at 4 °C for 7 h at 325 V. Denaturing PAGE was performed at 50 °C on 20% ($Os(bpy)_3^{3+}$ oxidative cleavage) or 8% (TRAP assay) gels containing 7 M urea. Electrophoresis running buffers were 0.5X (nondenaturing PAGE) with 100 mM KCl or 1X (denaturing PAGE) TBE and were prepared from a 10X TBE stock containing 0.89 M tris, 20 mM EDTA, and 0.89 M boric acid, pH 8.3. All gels were wrapped and placed on a phosphor screen, exposed overnight, scanned on a Molecular Dynamics Storm 840 phosphorimager, and analyzed using ImageQuaNT software.

Oxidative Cleavage of 8OG by $Os(bpy)_3^{3+}$. Stock solutions of ^{32}P-radiolabeled oligonucleotides **2** or **4** were prepared as G quartets or duplexes by combining 100 μL of ^{32}P-radiolabeled **2** or **4**, 40 μL of 100 μM unlabeled **2** or **4**, 25 μL of 1.6 M KCl, 40 μL of 100 mM KP_i, pH 7, and 15 μL of MilliQ water. Solutions also contained either an additional 80 μL of MilliQ water (G quartet **2** or **4**) or and additional 80 μL of a 100 μM stock solution of a shortened Watson−Crick complementary strand (duplex **2** or **4**). The complement was designed

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to give a three bp overhang that contains a single-stranded guanine on the 3′-end of the 8OG-containing oligonucleotides.[56] Samples were hybridized by heating to 90 °C for 5−10 min followed by slow cooling to room temperature over 2−3 h. Oxidative cleavage experiments were performed by combining 30 μL of the stock solutions of ^{32}P-radiolabeled G quartet or duplex **2** or **4** with 10 μL of a 60 μM stock solution of $Os(bpy)_3^{3+}$ in water and allowing the reaction to proceed for 5 min at room temperature. Samples were quenched, piperidine treated, and prepared for PAGE as described previously.[56] Final concentrations were 100 mM KCl, 10 mM KP_i, 10 μM **2** or **4**, and 15 μM $Os(bpy)_3^{3+}$. Solutions of duplex **2** or **4** contained a final concentration of 20 μM shortened Watson−Crick complement. Positions of guanines were determined by oxidative cleavage of **1** using flash-quench with $Ru(bpy)_3^{2+}$ and $Fe(CN)_6^{3-}$ as published previously.[56]

Telomeric Repeat Amplification Protocol (TRAP Assay). Oligonucleotides **TS** and **6**−**8** were ^{32}P-radiolabeled according to instructions in the TRAPeze telomerase detection kit. Briefly, 5 μL of gel-purified primer/substrate stock (approximately 0.1 μg/μL) was combined with 1 μL of 10X forward reaction buffer (Life Technologies), 2 μL of DEPC-treated water, 1 μL of T4 kinase, and 1 μL of [γ-^{32}P]-ATP. The mixture was incubated for 20 min at 37 °C followed by a 5 min incubation at 85 °C. The ^{32}P-radiolabeled primer/substrate generated in this manner was used directly for the TRAP assay. The master mix for the TRAP assay was prepared by combining 10X PCR buffer (Life Technologies), 50 mM $MgCl_2$ (Life Technologies), 50X dNTP mix (Intergen), primer mixture (Intergen), DEPC-treated water, and Taq polymerase (Life Technologies) in the appropriate amounts to generate final concentrations as outlined in the TRAPeze kit manual. We observed that Taq polymerase did not amplify telomeric products when 10X TRAP reaction buffer (Intergen) was used. The PCR protocol (Hybaid Sprint thermocycler) was one cycle at 30 °C for 20 min followed by 30 cycles of 94 °C for 30 s and 54 °C for 30 s. After the PCR program had completed, samples were transferred to 1.5-mL tubes and ethanol-precipitated with 3 M ammonium acetate and 1 μL of 250 mg/mL glycogen. The pellets were redissolved in 2 μL of 0.5X TBE and 14 μL of denaturing dye (98% formamide with bromophenol blue and xylene cyanol) before being loaded onto the gel. The gene for penicillin binding protein 5 used in a PCR amplification control experiment (see Supporting Information) was a gift from Dr. P. A. Ropp at the University of North Carolina at Chapel Hill.

Circular Dichroism. Circular dichroism (CD) measurements were collected with 1 mm path length quartz cuvettes using an Aviv circular dichroism spectropolarimeter, model 62DS. The wavelength range was 350−210 nm. Samples of unlabeled G quartet or duplex **2** or **4** were prepared as described above for electrochemistry and oxidative cleavage experiments except that the complementary strand was the shortened Watson−Crick complement in all cases. The CD spectra are the average of three scans. Comparable background scans without DNA were collected, averaged, and subtracted from the scans collected for solutions containing DNA.

Results

On the basis of our previous study of oxidative susceptibility of guanines in intramolecular G quadruplexes of telomeric DNA and reports that 8OG is observed in telomeric sequences,[44,45] we were interested in the structure and reactivity of telomeric repeats containing 8OG. Therefore, 8OG was substituted in sequence **1** to give oligonucleotides **2**−**5** in which the position of the 8OG in the GGG triplet was varied (Table 1). By site-specifically substituting 8OG for guanine, the reactivity of a single position in the telomeric sequence could be investigated as a function of structure *and* position in the GGG triplet. The oligonucleotides also were designed to probe the importance of the syn versus anti conformations of guanosine. The NMR structure of a sequence identical to **1** except without the TGT

Table 1. Oligonucleotide Sequences

	sequence (5′ → 3′)	8OG
1	AGG GTT $AG_{15}G_{14}$ G_{13}TT AG_9G_8 G_7TT AG_3G_2 G_1TG T	−
2	AGG GTT A*X*G GTT AGG GTT AGG GTG T[a]	+
3	AGG GTT AG*X* GTT AGG GTT AGG GTG T[a]	+
4	AGG GTT AGG GTT A*X*G GTT AGG GTG T[a]	+
5	AGG GTT AGG GTT AG*X* GTT AGG GTG T[a]	+
TS	AAT CCG TCG AGC AGA GTT	−
6	AAT CCG TCG AGC AGA GGG	−
7	AAT CCG TCG AGC AGA *X*GG[a]	+
8	AAT CCG TCG AGC AGA G*X*G[a]	+

[a] X = 7,8-dihydro-8-oxoguanine.

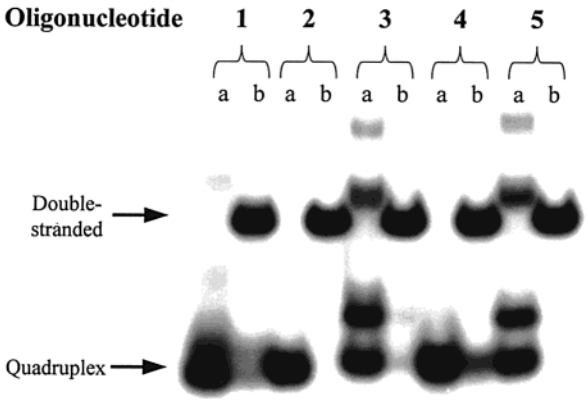

Figure 2. Phosphorimage of a 16% nondenaturing polyacrylamide gel of 8OG-containing telomeric sequences. All samples contain 100 mM KCl, 10 mM KP_i, pH 7. Lane a, G quadruplex; lane b, duplex. Note that the duplex contains a shortened Watson−Crick complement constructed to generate a single-stranded 3 bp overhang on the 3′-end. Multiple species are present in lane a for oligonucleotides **3** and **5**.

on the 3′-end showed that guanosines 8 and 15 both adopt syn conformations, while guanosines 9 and 14 adopt the anti conformation (numbering of guanines is shown in sequence **1**, Table 1).[7] This structure was performed in the presence of Na^+ (not K^+), but is likely relevant to that discussed here.

Because 8OG typically adopts an anti conformation,[65] we expected that **2** and **5**, which contain 8OG substituted for syn guanosines, would not assemble into intramolecular G quadruplexes. Figure 2 shows nondenaturing PAGE in the presence of 100 mM KCl of **1**−**5** in both intramolecular G quadruplex and double-stranded forms. For **1**, **2**, and **4**, only the intramolecular G quadruplex is observed. In contrast, for **3** and **5**, some intramolecular quadruplex appears to be formed, but another structural form that migrates faster than the double-stranded control also is formed. Thus, the position of the 8OG substitution in the sequence is more important in determining intramolecular G quadruplex formation than the syn versus anti guanosine conformation. A hydrogen bond in the G quadruplex plane is lost when 8OG replaces G. This loss of a hydrogen bond may be more important in the central than in the other G quadruplex planes, explaining why **3** and **5** do not fold into intramolecular quadruplexes (Figure 1).

The susceptibility of 8OG to one-electron oxidation in **2** and **4** was investigated by treatment of the oligonucleotides with the oxidant $Os(bpy)_3^{3+}$ followed by piperidine-induced strand scission. The $Os(bpy)_3^{3+}$ oxidant was chosen because its reduction potential (0.62 V vs Ag/AgCl[54]) is close to the

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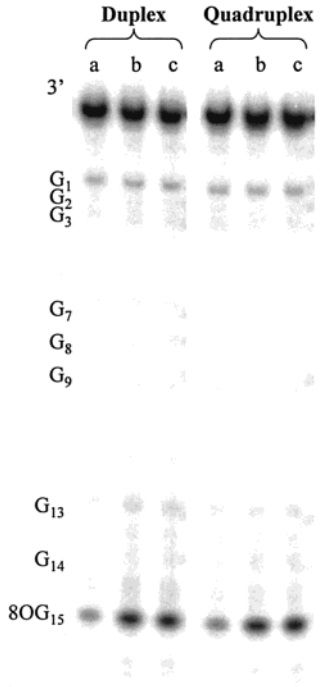

Figure 3. Phosphorimage of a 20% denaturing polyacrylamide gel showing $Os(bpy)_3^{3+}$ oxidation of 8OG in telomeric oligonucleotide **2**. All samples contained 100 mM KCl, 10 mM KP_i, pH 7, and 10 μM **2**. Duplex **2** was prepared by hybridization to 20 μM shortened Watson−Crick complement (see Figure 2 legend). Lane a, no $Os(bpy)_3^{3+}$; lanes b and c, 15 μM $Os(bpy)_3^{3+}$. Positions of guanines were determined by Maxam−Gilbert G reaction.

Table 2. Cleavage Intensities and Rate Constants for 8OG Oxidation in **2** and **4** by $Os(bpy)_3^{3+}$

sample	cleavage intensity[a]	rate constant (M^{-1} s^{-1})[b]
G quartet **2**	0.38 ± 0.04	$(6.2 \pm 1.7) \times 10^4$
B-form **2**	0.38 ± 0.01	$(3.0 \pm 1.3) \times 10^4$
G quartet **4**	0.31 ± 0.08	$(5.2 \pm 0.67) \times 10^4$
B-form **4**	0.21 ± 0.02	$(3.1 \pm 1.3) \times 10^4$

[a] Intensity determined as 8OG band relative to unreacted oligonucleotide. Intensity ratios are the average of three measurements. Average background cleavage intensity for the 8OG band without Os(III) was 0.11 ± 0.03. [b]Second-order rate constants determined by digital simulation of cyclic voltammograms as described in Materials and Methods. Rate constants are the average of at least three measurements and are per mole of 8OG.

reduction potential of 8OG (0.74 V vs NHE[66]). Oligonucleotides **2** and **4** were chosen because they assembled into a single structure based on nondenaturing PAGE. Figure 3 shows the oxidative cleavage induced by Os(III) for intramolecular G quadruplex and double-stranded **2**. For either structure, the 8OG at position 8 in **2** was the predominant site of damage when Os(III) was the oxidant. For **4**, the 8OG at position 14 was the major site of damage, which is not surprising given the structural similarity of **2** and **4** and the placement of the 8OG at the 5′-G position of a GGG triplet in both oligonucleotides. Quantitation of the 8OG cleavage intensities relative to unreacted oligonucleotide showed that the cleavage intensities were essentially the same regardless of whether the oligonucleotide was in the duplex or quadruplex form (Table 2). Changes in the concentration of oligonucleotide relative to oxidant concentration yielded the same result. We[55] and others[49] have observed previously that ratios for the relative reactivity of guanine or 8OG in various

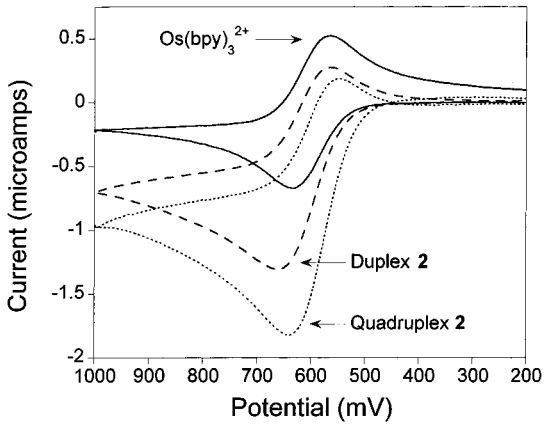

Figure 4. Cyclic voltammograms of 25 μM $Os(bpy)_3^{2+}$ collected at 25 mV/s in 800 mM NaCl, 50 mM NaP_i, pH 7 in the absence (—) and presence of 50 μM G quartet **2** (- - -) or 50 μM duplex **2** (– – – –). The Watson−Crick complement used in the duplex is full-length.

sequence contexts are smaller when oxidation is analyzed by denaturing PAGE than when measured using mediated electrochemistry. Therefore, the inability of oxidative cleavage followed by denaturing PAGE to differentiate 8OG reactivity in various structural contexts in this case is not surprising. The apparent decreased sensitivity of oxidative cleavage followed by denaturing PAGE is likely from chemistry that occurs after the initial one-electron oxidation step.

Mediated electrochemical oxidation of nucleic acids is not subject to ambiguities resulting from follow-up chemistry because the one-electron oxidation step is measured in real time.[55] We therefore turned to electrocatalytic oxidation of 8OG using $Os(bpy)_3^{2+}$ to differentiate the reactivity of 8OG in G quadruplex versus duplex **2** and **4**.[54] Replacing a specific guanine of interest with either a nonreactive inosine[55] or a more reactive nucleobase like 8OG[49,54] allows us to measure the one-electron oxidation properties of a sole site rather than the average reactivity of all guanines in the G quartet or duplex (Figure 1). A major difference between this study and previous work is that substitution of 8OG for guanine in this case probes reactivity as a function of multiple, specific environmental effects (i.e., global fold, sequence context, and nucleobase conformation). Cyclic voltammograms in Figure 4 show that the 8OG in duplex versus G quartet forms of **2** can be distinguished clearly from one another. The second-order rate constants for oxidation of 8OG in **2** or **4** differ by about a factor of 2 with the 8OG in the G quartet having a higher rate constant than the 8OG in B-form DNA (Table 2). This result is consistent with previous measurement of guanine reactivity in G quadruplex versus duplex forms[56] and shows that the ratios of the average rate constants are the same as those determined for the site-specifically substituted oligonucleotides.

Because samples prepared for oxidative cleavage and electrochemistry experiments were prepared under different conditions (i.e., 800 mM Na^+ for electrochemistry vs 100 mM K^+ for oxidative cleavage), we used circular dichroism spectroscopy to confirm that **2** and **4** generated the same G quadruplex or duplex structures under both sets of conditions (spectra shown in Supporting Information). Nondenaturing PAGE could not be used to confirm structure because electrochemistry experiments used 0.8 M NaCl, which is incompatible with nondenaturing PAGE. Positive features in the CD spectra at 240 and 290 nm

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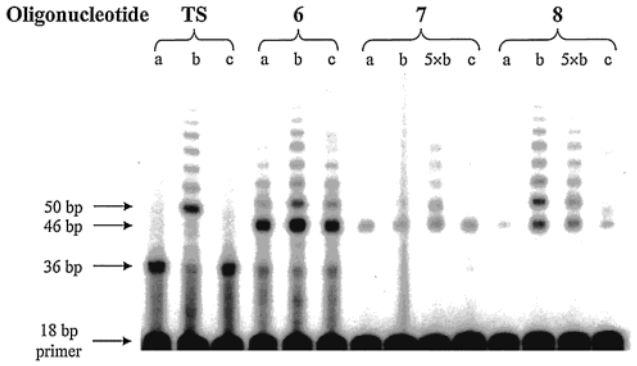

Figure 5. Phosphorimage of an 8% denaturing polyacrylamide gel showing products of the TRAP assay using the standard **TS** substrate or oligonucleotides **6**, **7**, and **8**. Lane a, lysis buffer control; lane b, telomerase-positive cell extract; lane 5 × b, like lane b, but with a 5-fold excess of substrate; lane c, heat-denatured telomerase-positive cell extract. The internal standard band at 36 bp is visible only in lanes containing the **TS** primer/substrate because the other primers are not exact complements and, therefore, do not promote efficient amplification of the internal standard sequence. The shift in position of the first band of telomeric products from 50 bp using **TS** to 46 bp using **6**, **7**, and **8** is due to substrate alignment on the RNA template and is discussed in the text.

and a negative feature at 260 nm show that **2** folds into an antiparallel quadruplex.[67] The intramolecular basket-type G quadruplex formed by 5′-d[$(AGGGTT)_3$AGGG]-3′, which is the parent oligonucleotide of **1**−**5**, is also an antiparallel quadruplex.[7] As expected, the duplex forms of the oligonucleotides show characteristic B-form features at 240 and 270 nm.[68] Regardless of whether 800 mM Na^+ or 100 mM K^+ were used, both **2** and **4** formed the desired structures, validating our use of low ionic strength for PAGE and high ionic strength for electrochemistry.

Finally, the 8OG-containing oligonucleotides were used as substrates for telomerase in the TRAP assay. The first three lanes of Figure 5 are control lanes showing telomerase activity using oligonucleotide **TS** under conventional TRAP conditions.[69] Lanes 4−6 show the results of the TRAP assay using **6**, which has been shown previously to generate telomeric products[70] and contains a GGG triplet at the 3′-end of the primer/substrate. The start band of **6** is shifted by four bases because the primer base pairs with the RNA template of telomerase in a register different from the **TS** primer.[70] Although lanes 4 (control) and 6 (heat-denatured telomerase-positive cell extract) indicate some mispriming with **6**, lane 5 with active telomerase-positive cell extract shows many more bands, indicating synthesis of telomeric repeats.

Changing the 5′-guanine in the GGG triplet to 8OG in **7** produces a significant decrease in the activity of telomerase (compare lanes 5 and 8). A 5-fold increase in the concentration of **7** does not produce telomerase activity comparable to that of **6** (compare lanes 5 and 9) but does give an increase relative to lane 8. In contrast, primer **8** in which 8OG has been placed in the middle of the GGG triplet does generate telomeric products (lane 12), even at a 5-fold higher concentration (lane 13). Quantitation of the telomeric product bands was not attempted because the modified oligonucleotides **6**−**8** are not exact complements to the internal control or the quantitation standard included with the kit, so variations in the PCR amplification efficiency are likely and would obscure the quantitative differences in telomerase activity.

Because the TRAP assay uses telomerase and Taq polymerase, we needed to determine that 8OG primers **7** and **8** inhibited telomerase only. We designed an experiment in which a primer with a GGG triplet at the 3′-end was used to amplify a 100 bp segment of the gene for penicillin-binding protein 5. Two other primers were designed in which 8OG was substituted in the 5′-G and middle positions of the GGG triplet. No inhibition of amplification was observed, indicating that Taq polymerase is not inhibited by primers containing 8OG in GGG sequences (data shown in Supporting Information).

Discussion

Nucleic Acid Structure. Our studies show that incorporation of 8OG into certain positions of telomeric DNA interferes with G quadruplex assembly and reduces the activity of telomerase. When 8OG replaces a 5′-G in a GGG triplet (oligonucleotides **2**, **4**, and **7**), the telomeric sequence folds into an intramolecular G quadruplex, and telomerase activity is reduced. In contrast, substitution of 8OG in the middle of a GGG triplet (**3**, **5**, and **8**) disrupts intramolecular folding into a G quadruplex and produces no obvious change in telomerase activity. Therefore, replacement of 8OG for G alters the structure or affinity of telomeric DNA for telomerase, interfering with telomerase binding and extension of single-stranded telomeric DNA. The reduced telomerase activity we observed with **2**, **4**, and **7** is consistent with previous measurements of telomerase activity in the presence of telomeric DNA that forms G quadruplexes.[19] In contrast, the ability of telomerase to utilize a substrate with the site-specific substitution of 8OG for guanine has not been previously reported.

The poor telomerase activity of substrate **7** could result from formation of an intermolecular, four-stranded G quadruplex. In this case, the intermolecular G quadruplex would need to dissociate before binding of telomerase and extension of the substrate.[71] This mode of inhibition by **7** is unlikely because the TRAP assay uses very low concentrations of **7**, which should decrease the possibility of intermolecular association. Further, a 5-fold increase in the concentration of **7** in the TRAP assay produces some telomerase activity (Figure 5), showing that telomerase binding to single-stranded **7** competes effectively with intermolecular G quadruplex formation.

Electron-Transfer Chemistry. The rate of one-electron oxidation for nucleobases is related to both sequence-specific stacking and accessibility of the base to the exogenous oxidant.[49,50,52,55] Typically, the oxidation susceptibility of 8OG is measured by exposure of 8OG-containing oligonucleotides to oxidants, followed by quantification of piperidine-induced strand-scission products.[49] In our case, oxidative cleavage analyzed by denaturing PAGE was not sensitive enough to distinguish 8OG reactivity in intramolecular quadruplexes from that observed for 8OG in B-form telomeric sequences, most likely due to chemistry that occurs after the initial oxidation

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step. Therefore, we turned to real-time measurements of one-electron oxidation of 8OG in telomeric DNA using mediated electrocatalysis. The rate constant for one-electron oxidation of 8OG in intramolecular G quadruplexes is a factor of 2 higher than the rate constant for oxidation of 8OG in duplex DNA. The rate constant for oxidation of 8OG in duplex DNA reported here (3×10^4 M^{-1} s^{-1}, Table 2) agrees well with that published previously for 8OG in a duplex (2.2×10^4 M^{-1} s^{-1}).[54] As expected, the rate constant for oxidation of 8OG in quadruplex DNA has a value that is between that found for duplex DNA and that reported for single-stranded oligonucleotides containing 8OG (1.4×10^6 M^{-1} s^{-1}).[54] A similar trend was observed for guanines in single-stranded, quadruplex, and duplex telomeric DNA.[56]

Substituting 8OG for a single guanine in telomeric DNA and preserving the global DNA structure (quadruplex or duplex) allowed us to determine the relative one-electron oxidation rates at a single site as a function of structure, rather than the *average* guanine oxidation rate constant for all of the guanines in the telomeric sequence.[56] The choice of $Os(bpy)_3^{3+}$ as the one-electron oxidant ensures that only the oxidation susceptibility of a single 8OG is probed because guanine is not oxidized by Os(III). Such selective replacement of 8OG for guanine has been employed previously to investigate base-stacking effects on oxidation chemistry in guanine doublets in ordinary B-form DNA.[49] The ability to differentiate the relative reactivities of site-specifically substituted 8OGs suggests a general strategy for probing site-specific trends in oxidation chemistry in diverse biomolecular structures. Such a strategy could involve minimally modified building blocks that are oxidatively reactive and substituted site-specifically into diverse structures in cases where native structure is preserved.

Biological Implications. The in vivo consequence of reduced telomerase activity in cells with 8OG-containing telomeres has yet to be determined. However, skin cells are the most susceptible to damage from UVA irradiation and, therefore, are likely to contain 8OG in their telomeres; Kim et al.[23] have reported that telomerase activity was detected in all samples of skin cancer tested, while normal skin cells exhibited no telomerase activity. Taking these findings together with our results, telomerase should be competent in UV-damaged skin cells that contain 8OG in the middle of telomeric GGG triplets. The activity of telomerase in skin cancers could therefore be related to the ability of telomerase to use chemically damaged substrates.

The structural effects of 8OG in telomeric sequences are significant because if 8OG formation in telomeres occurs in vivo in significant yield and with sufficient frequency, the therapeutic effects of drugs that rely on intact telomeric sequences to inhibit telomerase may be compromised. Guanine quadruplex-stabilizing ligands may or may not induce telomerase inhibition in 8OG-containing telomeric sequences. It is possible that these drugs may promote intramolecular G quadruplex formation in otherwise nonfolding 8OG-containing telomeric sequences, resulting in telomerase inhibition. However, it is also possible that the G quadruplex-stabilizing ligands will show decreased efficacy depending on the position of 8OG in a telomeric sequence because, as our findings here show, telomeres containing a single 5′-GXG-3′ (X = 8OG) triplet do not assemble exclusively into intramolecularly folded guanine quadruplexes. Alternatively, the stabilization of the quadruplex and the telomere shortening caused by oxidation may produce an additive effect and increase the effectiveness of the inhibitor. Drugs based on nucleotide analogues or reverse transcriptase inhibitors would not be expected to be affected as drastically by oxidation of G to 8OG in telomeres.

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Supporting Information Available: Circular dichroism spectra of **2** and nondenaturing polyacrylamide gel showing PCR amplification of a template using 8OG-containing primers and Taq polymerase (PDF). This information is available free of charge on the Internet at http://pubs.acs.org.

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