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Spontaneous DNA Lesions Modulate DNA Structural Transitions Occurring at Nuclease Hypersensitive Element III_1 of the Human c-myc Proto-Oncogene

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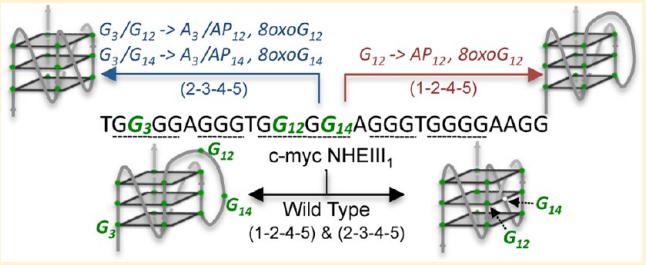
ABSTRACT: G quadruplex (G4) DNA is a noncanonical four-stranded DNA structure that can form in G repeats by stacking of planar arrays of four hydrogen-bonded guanines called G quartets, in the presence of potassium ions. In addition to a presumed function in the regulation of gene expression, G4 DNA also localizes to regions often characterized by genomic instability. This suggests that formation of this structure may interfere with DNA transactions, including processing of DNA damage at these sites. Here we have studied the effect of two spontaneous DNA lesions, the abasic site and 8-oxoguanine, on the transition from duplex to quadruplex DNA structure occurring at nuclease hypersensitive element III_1 ($NHEIII_1$) of the human c-myc promoter. We show by dimethyl sulfate footprinting and RNA polymerase arrest assays that at physiological concentrations of potassium ions $NHEIII_1$ folds into two coexisting G4 DNA structures, myc-1245 and myc-2345, depending on which G runs are utilized for G quartet formation. We found that a single substitution of G12 of $NHEIII_1$ with a single abasic site or a single 8-oxoguanine prevented formation of G4 structure myc-2345 in favor of structure myc-1245, where the lesion was accommodated in a DNA loop formed by G11-AP12/(or 8-oxoG12)-G13-G14. Surprisingly, when an additional G to A base substitution was introduced at position 3 of $NHEIII_1$, we observed formation of myc-2345. The extent of this structural transition was modulated by the location and type of lesion within the G11−G14 repeat. Our data indicate that spontaneous lesions formed in the G4-forming sequence of c-myc $NHEIII_1$ affect the structural transitions occurring at this regulatory site, potentially altering transcription factor binding and DNA repair of lesions formed in this highly regulated sequence.



Excision DNA repair pathways are essential for the maintenance of genomic stability and work by removing deleterious DNA lesions that would otherwise result in mutations, leading to human disease.[1−4] Little is known about the efficiency and fidelity of excision repair pathways in genomic regions that can assume noncanonical DNA structures, which have been recently shown to correspond to sites frequently mutated in human disease and cancer. An increased level of exposure to mutagenic agents at sites of unusual structures combined with impaired recognition and repair of DNA damage by the DNA repair machinery at these sites may render these regions particularly susceptible to mutations. Genetic studies indicate that excision repair processes are implicated in the mutagenesis associated with these sites.[5−12] Furthermore, transcription through these regions plays a central role in promoting the formation of these noncanonical structures and in the associated mutagenesis.[12−15]

It has been shown that increased levels of CAG triplet repeat expansions occur when base excision repair of oxidative damage occurs at or near the repeat sequence.[16] The bacterial nucleotide excision repair complex UvrABC recognizes and cleaves triplex DNA structures formed in the polycystic kidney disease 1 gene, resulting in a high mutation frequency in that gene.[6] Furthermore, it is known that transcription through these regions plays a central role in promoting the formation of these structures and in the mutagenesis associated with these sites.[12−15] In mammalian cells, triplex DNA formation results in spontaneous mutations that are dependent on nucleotide excision repair and transcription-coupled repair (TCR) factors XPA and CSB.[17,18] Furthermore, it was shown that transcription through CAG repeats in human cells increases the level of repeat contraction ∼15-fold in both confluent and proliferating cells: repeats were stabilized against contraction by siRNA knockdown of CSB, which is required for TCR, but not by siRNA knockdown of XPC, required only for global genomic repair.[12,15]

Although these observations suggest that excision repair pathways are involved in the observed instability of repetitive sequences, the molecular events that characterize this interaction and are responsible for genetic changes remain to be investigated. In addition, little is known about the effect that

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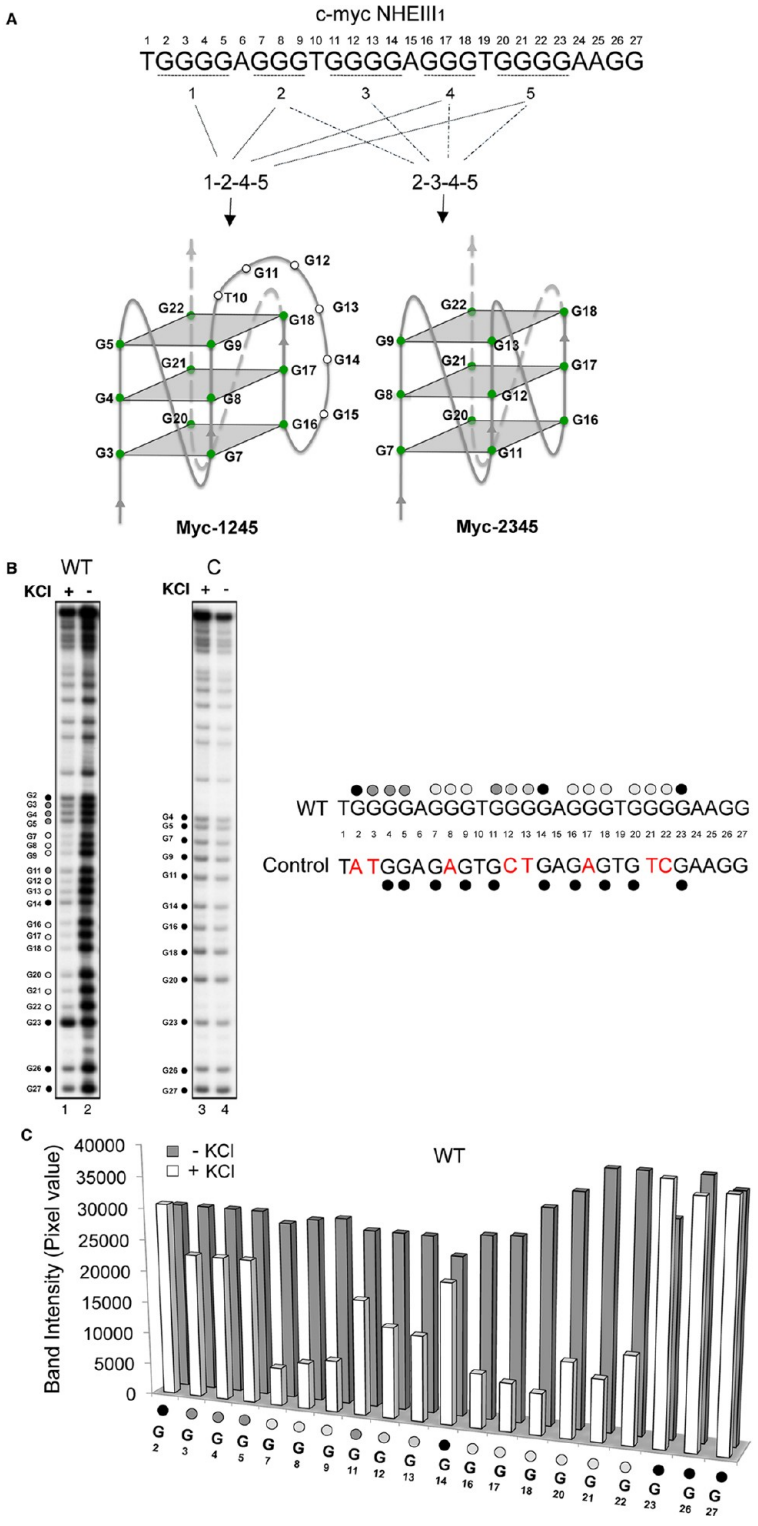

Figure 1. DNA sequences used in this study. (A) Sequence of nuclease hypersensitive element III_1 from the c-myc gene. Wild-type (wt) $NHEIII_1$ can fold into two G4 conformations, myc-1245 and myc-2345, depending on which G repeat is utilized in G4 DNA formation. Bases are numbered according to the sequence of $NHEIII_1$. (B) Dimethyl sulfate (DMS) footprinting experiment showing G4 formation in wt $NHEIII_1$. As a negative control, we utilized a sequence identical to that of wt $NHEIII_1$ except for single-base substitutions (G2, G8, G13, G17, G21, and G22 changed to A2, A8, T13, A17, T21, and C22, respectively) resulting in interruptions in the G runs, thus yielding a G-rich sequence without the ability to fold into G4. DNA substrates for DMS footprinting consisted of 91-mer oligonucleotides labeled at the 5′ end with ^{32}P. These substrates were incubated from 95 °C to room temperature in the presence or absence of 100 mM KCl to generate DNA oligonucleotides in which the G4-forming sequence was folded (lane 1) or not folded (lane 2) into G4 structure. G residues protected from DMS methylation are marked with an open circle; those that are not protected are marked with a filled circle, and those that are partially protected are marked with a gray circle. (C) Quantitation of the DMS data. The intensity of the bands corresponding to G2−G27 was measured by phosphorimage analysis as described in Experimental Procedures.

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Table 1. Sequences Used in This Study

T7 primer	5′-TAATACGACTCACTATA-3′
wt	5′-GCTACATGCTCTAGATCT/GGAAGGGGTGGGAGGGGTGGGAGGGGT/ CTGCACCCAGCTGCTTCGTCCGAAGACCC*TATAGTGAGTCGTATTA*-3′
control	5′-GCTACATGCTCTAGATCT/GGAAGCTGTGAGAGTCGTGAGAGGTAT/ CTGCACCCAGCTGCTTCGTCCGAAGACCC*TATAGTGAGTCGTATTA*-3′
A3	5′-GCTACATGCTCTAGATCT/GGAAGGGGTGGGAGGGGTGGGAGGAGT/ CTGCACCCAGCTGCTTCGTCCGAAGACCC*TATAGTGAGTCGTATTA*-3′
A12	5′-GCTACATGCTCTAGATCT/GGAAGGGGTGGGAGGAGTGGGAGGGGT/ CTGCACCCAGCTGCTTCGTCCGAAGACCC*TATAGTGAGTCGTATTA*-3′
AP12	5′-GCTACATGCTCTAGATCT/GGAAGGGGTGGGAGGAbasicGTGGGAGGGGT/ CTGCACCCAGCTGCTTCGTCCGAAGACCC*TATAGTGAGTCGTATTA*-3′
8-oxoG12	5′-GCTACATGCTCTAGATCT/GGAAGGGGTGGGAGG8-oxoGGTGGGAGGGGT/ CTGCACCCAGCTGCTTCGTCCGAAGACCC*TATAGTGAGTCGTATTA*-3′
A3/A12	5′-GCTACATGCTCTAGATCT/GGAAGGGGTGGGAGGAGTGGGAGGAGT/ CTGCACCCAGCTGCTTCGTCCGAAGACCC*TATAGTGAGTCGTATTA*-3′
A3/AP12	5′-GCTACATGCTCTAGATCT/GGAAGGGGTGGGAGGAbasicGTGGGAGGAGT/ CTGCACCCAGCTGCTTCGTCCGAAGACCC*TATAGTGAGTCGTATTA*-3′
A3/8-oxoG12	5′-GCTACATGCTCTAGATCT/GGAAGGGGTGGGAGG8-oxoGGTGGGAGGAGT/ CTGCACCCAGCTGCTTCGTCCGAAGACCC*TATAGTGAGTCGTATTA*-3′
A3/A14	5′-GCTACATGCTCTAGATCT/GGAAGGGGTGGGAAGGGTGGGAGGAGT/ CTGCACCCAGCTGCTTCGTCCGAAGACCC*TATAGTGAGTCGTATTA*-3′
A3/AP14	5′-GCTACATGCTCTAGATCT/GGAAGGGGTGGGAAbasicGGGTGGGAGGAGT/ CTGCACCCAGCTGCTTCGTCCGAAGACCC*TATAGTGAGTCGTATTA*-3′
A3/8-oxoG14	5′-GCTACATGCTCTAGATCT/GGAAGGGGTGGGA8-oxoGGGGTGGGAGGAGT/ CTGCACCCAGCTGCTTCGTCCGAAGACCC*TATAGTGAGTCGTATTA*-3′

DNA lesions exert on the formation and stability of noncanonical DNA structures. Here we have investigated whether two spontaneous DNA lesions, the abasic site and 8-oxoguanine, alter the transition to G4 DNA occurring in nuclease hypersensitive element III_1 ($NHEIII_1$) from the c-myc gene. This G4-forming sequence is composed of a polypurine/polypyrimidine tract that can undergo the transition from duplex to quadruplex DNA structure in vivo.[19] It consists of five G repeats that can fold into two G4 structures, myc-1245 and myc-2345, depending on which G repeat is utilized in the structure (Figure 1A). This G4-forming sequence is particularly relevant for studying mechanisms of mutagenesis because it localizes near the most frequent translocation hot spot for the c-myc gene in B-cell malignancies.[20−22] It was therefore of interest to elucidate the effect of the presence of two spontaneous DNA lesions, the abasic site and 8-oxoguanine, on the structural transitions occurring at this site.

EXPERIMENTAL PROCEDURES

Proteins and Reagents. T7 RNA polymerase (T7 RNAP) was purchased from Promega. Proteinase K was from Invitrogen. DNA oligonucleotides were purchased from Midland. Highly purified NTPs were purchased from Amersham Pharmacia Biotech. Radiolabeled nucleotides were from MP Biomedical. Piperidine and dimethyl sulfate (DMS) were from Sigma. Restriction enzymes were from New England Biolabs.

Preparation of DNA Templates for Transcription. Synthetic DNA templates for transcription reactions with T7 RNAP consisted of DNA oligonucleotides in which a double-stranded T7 promoter region was generated by annealing a 17-mer oligonucleotide to a sequence complementary to the T7 promoter sequence at the 3′ end of the single-stranded oligonucleotide. The oligomer sequences are listed in Table 1. T7 transcription substrates were obtained by incubating 10 pmol of T7 primer with 10 pmol of 91-mer oligonucleotide in a final volume of 10 μL for 3 min at 95 °C, which was followed by slow cooling to 37 °C. After the annealing step, samples were kept at −20 °C. To induce G4 DNA formation, which is dependent on the presence of potassium ions,[23] the annealing reaction was conducted in 100 mM KCl. Control samples were incubated in 100 mM LiCl or in TE buffer.

T7 RNAP Transcription Reactions. The DNA templates at a final concentration of 0.1 μM were incubated at 37 °C in a mixture of 50 units of T7 RNAP, 40 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 2 mM spermidine, 10 μCi of [α-^{32}P]GTP, 10 mM dithiothreitol, 212 units of RNAsin, 200 μM ATP, CTP, and UTP, and 20 μM GTP. Incubation continued at 37 °C for 30 min. Reactions were stopped by addition of 5 μg of proteinase K, 1% SDS, 100 mM Tris-HCl (pH 7.5), 50 mM EDTA, and 150 mM NaCl, followed by incubation for 15 min at room temperature. The nucleic acids were precipitated with ethanol, resuspended in formamide dye, and denatured at 90 °C for 3 min. The transcription products were resolved on a 12% denaturing polyacrylamide gel in Tris borate-EDTA containing 7 M urea. Gels were dried and autoradiographed using intensifying screens. Transcripts were quantified using a Typhoon phosphorimager and ImageQuant from GE Healthcare. The extent of arrest at the c-myc-1245 or -2345 structure was calculated by dividing the intensity of the 30−31- or 35−36-nucleotide transcript band by the sum of the intensity of the arrested and runoff RNA bands. The extent of arrest was adjusted by the G content of the RNA transcripts.

Dimethyl Sulfate Footprinting Assays. First, 2.5 pmol of 91-mers containing the G4-forming sequence was end-labeled with [γ-^{32}P]ATP and polynucleotide kinase. They were then heat denatured at 95 °C followed by slow cooling to room temperature in TE buffer in the presence or absence of 100 mM KCl. The 91-mers were cooled to 4 °C before being added to a solution containing 50 mM sodium cacodylate (pH 7.0) and 1 mM EDTA (pH 8.0). Five microliters of dymethyl sulfate was added and left to react for 5 min at room temperature. The reactions were stopped by addition of 1.5 M sodium acetate (pH 7.0), 1 M β-mercaptoethanol, and 1 μg/mL tRNA. DNA was precipitated with ethanol and resuspended in 1 M piperidine. After cleavage at 90 °C for 30 min, reactions were stopped when the mixtures were chilled in ice followed by

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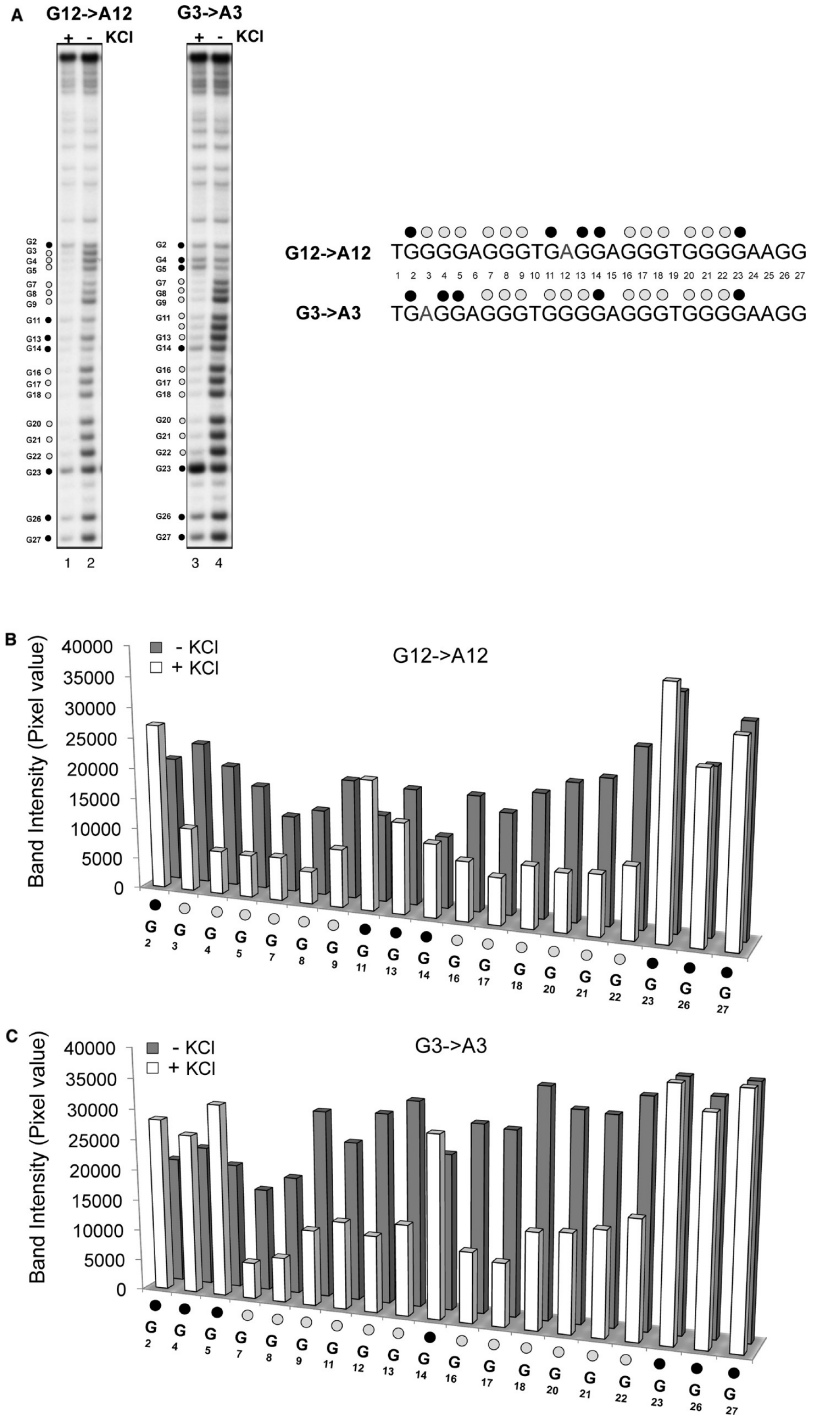

Figure 2. Single G to A substitutions in the c-myc G repeat promote selective formation of the c-myc-1245 or c-myc-2345 G4 structure. (A) DMS footprinting showing formation of the c-myc-1245 conformation when G12 is substituted with A12 (left) or the myc-2345 conformation when G3 is substituted with A3 (right). c-myc DNA substrates for DMS footprinting consisted of 91-mer oligonucleotides labeled at the 5′ end with ^{32}P. Incubation from 95 °C to room temperature in the presence or absence of 100 mM KCl generated substrates in which the G4-forming sequence folded (lanes 1 and 3) or did not fold (lanes 2 and 4) into G4 structure, as indicated by protection from DMS footprinting. G residues protected from DMS methylation are marked with empty circles, and those that are not protected are marked with filled circles. (B and C) Quantitation of the DMS data. The intensity of the bands corresponding to G2−G27 was measured by phosphorimager analysis as described in Experimental Procedures: (B) G12 → A12 mutant and (C) G3 → A3 mutant.

ethanol precipitation. The samples were resuspended in 100 μL of water and dried overnight in a speed vac concentrator. Samples were resuspended in 4 μL of formamide dye followed by denaturation for 3 min at 90 °C. The DNA samples were separated on a 12% denaturing polyacrylamide gel in Tris borate-EDTA containing 7 M urea. Gels were dried and autoradiographed using intensifying screens. G band intensities were quantified using a Typhoon phosphorimager and ImageQuant from GE Healthcare. For each sequence analyzed, the DNA fragments corresponding to DMS methylation of G26 and G27, which are located outside of the c-myc repeat, were

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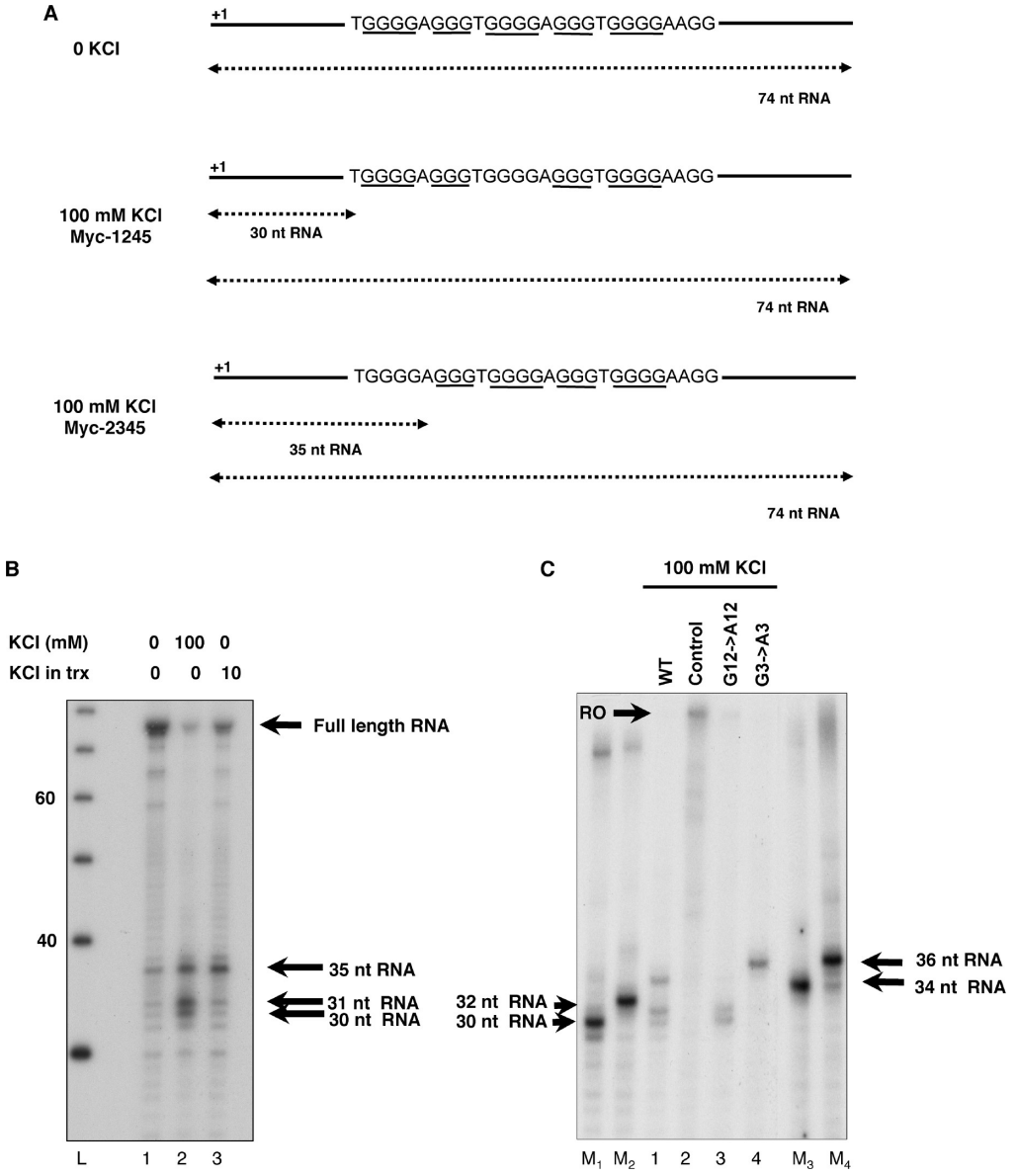

Figure 3. Detection of G4 DNA formation in the $NHEIII_1$ repeat by a transcription stop assay. (A) Schematic representation of transcription substrates containing the $NHEIII_1$ sequence and the expected sizes of the transcripts resulting from arrest at myc-1245 (30-nucleotide RNA) or myc-2345 (35-nucleotide RNA) structure. (B) Denaturing polyacryamide gel (12%) showing RNA transcripts from transcription of the wt c-myc $NHEIII_1$ sequence under different salt conditions; DNA templates were transcribed in vitro such that the transcripts were radioactively labeled. Elongation was allowed to proceed for 30 min at 37 °C after addition of NTPs to the reaction mixture, followed by RNA purification and separation on 12% denaturing polyacrylamide gels. Lane L contained the 10 bp DNA ladder. (C) Denaturing polyacryamide gel (12%) showing RNA transcripts from transcription of the wt c-myc $NHEIII_1$ sequence and single-base mutant sequences after incubation in the presence of 100 mM KCl. Each mutant was selected to generate no structure (control), myc-1245 structure (G12 → A12), or myc-2345 structure (G3 → A3). Lanes M_1–M_4 contained 30-, 32-, 34-, and 36-nucleotide RNAs synthesized from transcription of 47-, 49-, 51-, and 53-mer oligonucleotide DNA templates, respectively, with sequence identical to that of the c-myc-containing substrate. Lane L contained the 10 bp DNA ladder. Sites of transcription arrest are marked with arrows. RO denotes runoff RNA.

used for normalization between lanes treated with KCl and lanes left untreated.

RESULTS

Formation of G Quadruplex DNA in Nuclease Hypersensitive Site III_1 from the Human c-myc Proto-Oncogene under Physiological Conditions. We have started our analysis by investigating G4 DNA formation in a well-characterized G quadruplex-forming sequence, nuclease hypersensitive element III_1 ($NHEIII_1$) contained in the promoter of the c-myc proto-oncogene. This G-rich repeat is particularly relevant for studying mechanisms of tumorigenesis because it localizes near a frequent mutation hot spot for the c-Myc gene in human tumors.[20−22] This sequence is composed of a polypurine/polypyrimidine tract that can undergo a transition from duplex to quadruplex DNA structure in vivo[19,23−25] (Figure 1). Two G4 structures, c-myc-1245 and c-myc-2345, have been identified, depending on which G repeat is utilized in the structure (Figure 1A).[26−29] We directly tested G4 DNA formation in c-myc $NHEIII_1$ by dimethyl sulfate (DMS) footprinting experiments, a technique routinely used to identify G4 DNA formed in single-stranded synthetic

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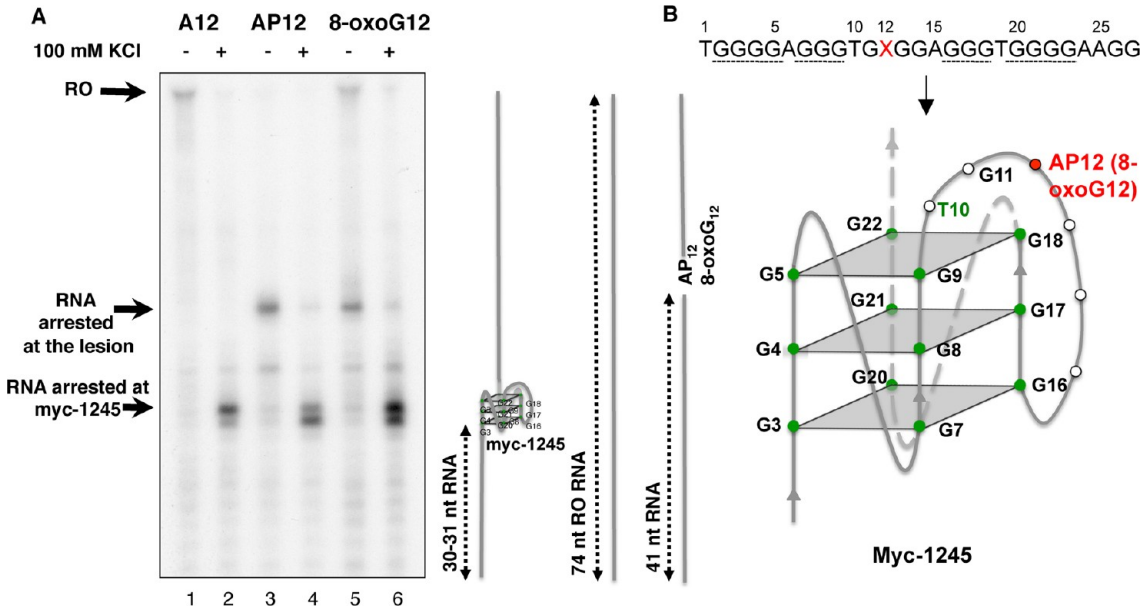

Figure 4. A single abasic site or a single 8-oxoguanine at position 12 of the c-myc G repeat promote formation of myc-1245 structure. Polyacryamide gel (12%) showing RNA transcripts from transcription of DNA substrates containing a base substitution (G12 → A12) or an abasic site or an 8-oxoguanine at position 12. Transcription was conducted after incubation of the transcription substrates in the absence (lanes 1, 3, and 5) or presence (lanes 2, 4, and 6) of 100 mM KCl. Lanes 1 and 2 contained $NHEIII_1$ containing a G to A base substitution at position 12, predicted to generate myc-2345 structure. Lanes 3 and 4 contained $NHEIII_1$ containing a single AP site at position 12. Lanes 5 and 6 contained $NHEIII_1$ containing a single 8-oxoG at position 12. Sites of transcription arrest are marked with arrows. RO denotes runoff RNA. (B) Predicted G4 structure of the AP or 8-oxoG12 $NHEIII_1$ sequence based on the transcription data shown in panel A.

oligonucleotides in vitro.[19,23,30] This assay is based on the property of DMS attacking N7 of guanine, which is accessible to DMS methylation when it is present in single-stranded or duplex DNA but not when it is paired to the exocyclic amino group of a neighboring guanine in a G quartet.[31,32] As a result, the Gs in the quartet are protected from methylation and subsequent piperidine cleavage compared to the unstructured control, resulting in a decreased intensity or the absence of the corresponding G band after DNA separation on sequencing gels.

When a 91-mer oligonucleotide containing c-myc $NHEIII_1$ was treated with DMS and piperidine, followed by 12% denaturing polyacrylamide gel electrophoresis (Figure 1B, lane 2), we observed bands with similar intensities corresponding to the Gs present in the G repeat and flanking sequences. However, when the c-myc repeat was incubated with 100 mM KCl prior to DMS treatment, we observed DMS protection of several Gs within the c-myc repeat (Figure 1B, lane 1). Protection of G7−G9, G16−G18, and G20−G22 is expected from formation of either myc-1245 or myc-2345. Protection of G11−G13 is expected only if myc-2345 forms. Protection of G3−G5 is expected only if myc-1245 forms. Depending on the extent of formation of one structure relative to the other in wt c-myc $NHEIII_1$, various degrees of protection of G11−G13 and G3−G5 would be expected. Consistent with the formation of both structures, we detected partial protection of G3−G5 and G11−G13 (Figure 1B,C). As a negative control, we utilized an identical sequence in which we interrupted the G runs with G → A and G → T substitutions (Figure 1B), resulting in a G-rich sequence that could not fold into G4 structure. As expected, no protection from DMS methylation was observed in this sequence either in the presence or in the absence of KCl (Figure 1B, lanes 3 and 4).

Single-Base Substitutions in the c-myc G Repeat Lead to the Formation of the c-myc-1245 Structure. The two G4 structures of the $NHEIII_1$ sequence consist of three G quartets that utilize G repeats 2, 4, and 5 (Figure 1). The myc-2345 structure forms when G repeat 3 participates in the G quartets, while the myc-1245 structure forms when G repeat 1 participates in the quartets and repeat 3 forms a six-base loop, T10-G11-G12-G13-G14-G15.[26−29] These structural requirements predict that base substitutions in G repeat 1 or 3 will be critical in promoting the transition either to myc-2345 or to myc-1245 structure. To test this prediction, we have introduced a single G → A base substitution at position 3 of repeat 1 or at position 12 of repeat 3 of oligonucleotides containing the c-myc $NHEIII_1$ sequence and conducted DMS footprinting experiments after incubation in the absence or presence of 100 mM KCl to induce the formation of G4 structure (Figure 2). We found that incubation with 100 mM KCl of the c-myc oligonucleotide containing a G3 → A3 base substitution generated DMS protection of G7−G9, G11−G13, G16−G18, and G20−G22 (Figure 2A, lane 3, and Figure 2C). Furthermore, G2, G4, and G5 did not show protection from DMS methylation. This pattern of DMS methylation is consistent with formation of myc-2345 structure. When DMS protection experiments were conducted with the oligonucleotides containing a single G12 → A12 base substitution, we observed DMS protection on the KCl-treated oligo at G3−G5, G7, G8, G16−G18, and G20−G23 (Figure 2A, lane 1, and Figure 2B). G11, G13, and G14 did not show DMS protection (Figure 2B), as expected from the predicted location of these Gs in the G11-A12-G13-G14 loop (Figure 1A). This pattern of DMS methylation is consistent with formation of myc-1245 structure.

G4 DNA Detection in c-myc $NHEIII_1$ by RNA Polymerase Transcription Stop Assays. Abasic sites and 8-oxoG are

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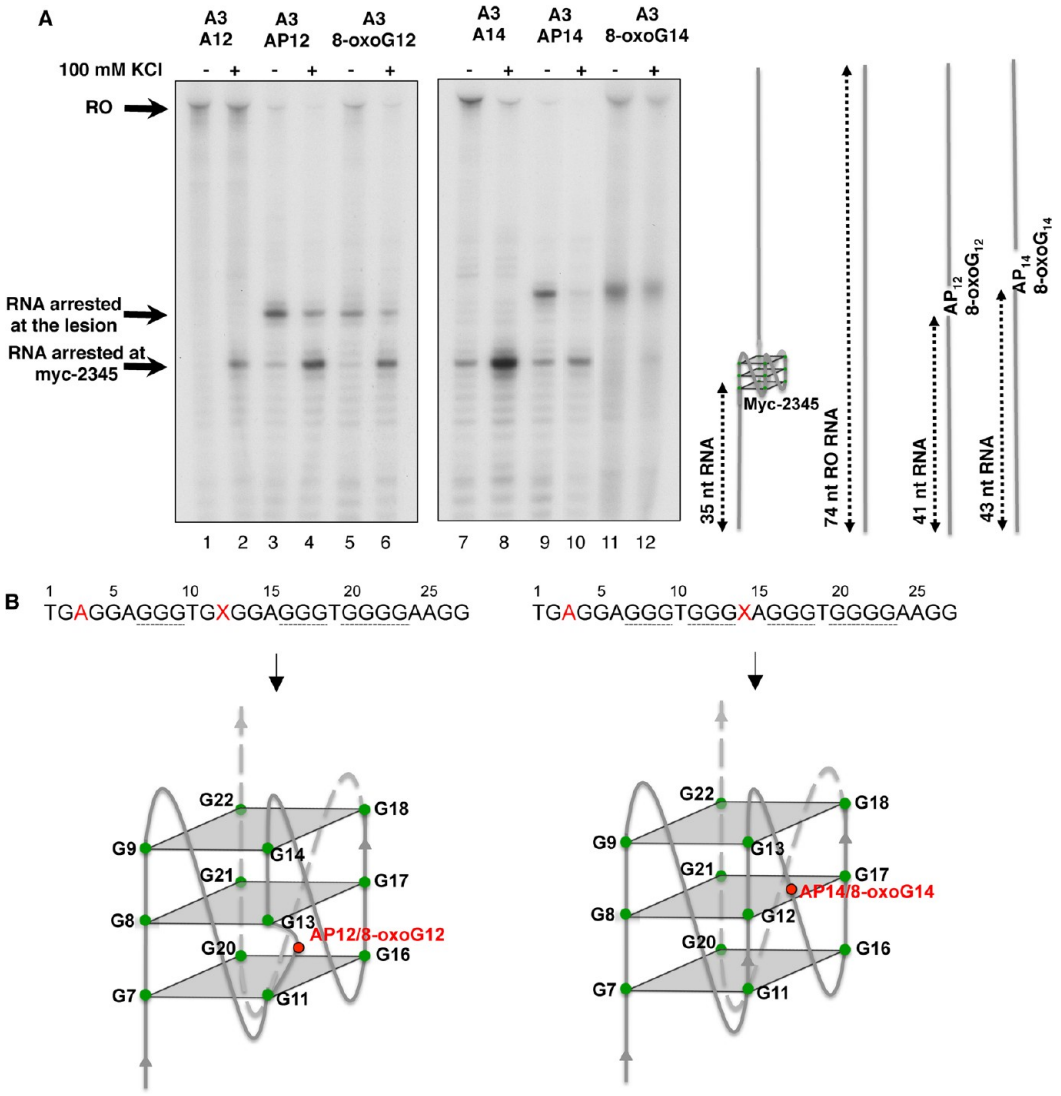

Figure 5. G3 → A3 base substitution in the c-myc repeat in addition to an abasic site or an 8-oxoG at position 12 or 14 that promotes the formation of c-myc-2345 structure. (A) Polyacrylamide gel (12%) showing RNA transcripts from transcription of DNA substrates containing a double base substitution (A3 → G3 and G12 → A12) or an abasic site or an 8-oxoguanine at position 12 or 14. Transcription was conducted after incubation of the transcription substrates in the absence (lanes 1, 3, 5, 7, 9, and 11) or presence (lanes 2, 4, 6, 8, 10, and 12) of 100 mM KCl. Lanes 1 and 2 contained $NHEIII_1$ containing a G to A base substitution at positions 3 and 12, predicted to interfere with myc-1245 and myc-2345 structures. Lanes 3 and 4 contained $NHEIII_1$ containing a G → A base substitution at position 3 and a single AP site at position 12. Lanes 5 and 6 contained $NHEIII_1$ containing a G → A base substitution at position 3 and a single 8-oxoG at position 12. Lanes 7 and 8 contained $NHEIII_1$ containing a G to A base substitution at positions 3 and 14, predicted to interfere with myc-2345 structure. Lanes 9 and 10 contained $NHEIII_1$ containing a G → A base substitution at position 3 and a single AP site at position 14. Lanes 11 and 12 contained $NHEIII_1$ containing a G → A base substitution at position 3 and a single 8-oxoG at position 14. Sites of transcription arrest are marked with arrows. RO denotes runoff RNA. A schematic representation of the expected transcript sizes is shown at the right. (B) Possible G4 structures of the A3/AP12,14 or A3/8-oxoG12,14 $NHEIII_1$ sequences derived from the transcription data shown in panel A.

sensitive to piperidine treatment, making it problematic to utilize DMS protection assays to detect tetraplex formation in the damage-containing oligodeoxyribonucleotides.[33] As an alternative approach, we have applied an in vitro transcription stop assay we have previously shown to be very effective in identifying and quantifying G4 DNA structure formation in the human c-myb gene.[34] This assay takes advantage of the ability of T7 RNA polymerase to synthesize RNA transcripts utilizing single-stranded DNA molecules as substrates, when a double-stranded promoter region is provided to initiate transcription, and of the unique property of G4 DNA to form in single-stranded DNA in the presence of KCl, which stabilizes G quartets. In this assay, G4 DNA formation is detected as premature blockage of T7 transcription and the resulting synthesis of RNAs shorter than the full-length runoff transcripts (Figure 3A). Because we know the location of c-myc $NHEIII_1$ in our transcription templates, we can carefully determine where transcription was arrested with respect to the non-B DNA structure. Furthermore, by measuring the extent of arrested transcript with respect to full-length RNA, we can quantitate the extent of the transition to quadruplex DNA. Transcription of the c-myc $NHEIII_1$ oligonucleotide preincubated in the absence of KCl generated mostly a 74-nucleotide RNA, as expected from transcription up to the end of the oligonucleotide (Figure 3A and Figure 3B, lane 1). When transcription was conducted after preincubation of the

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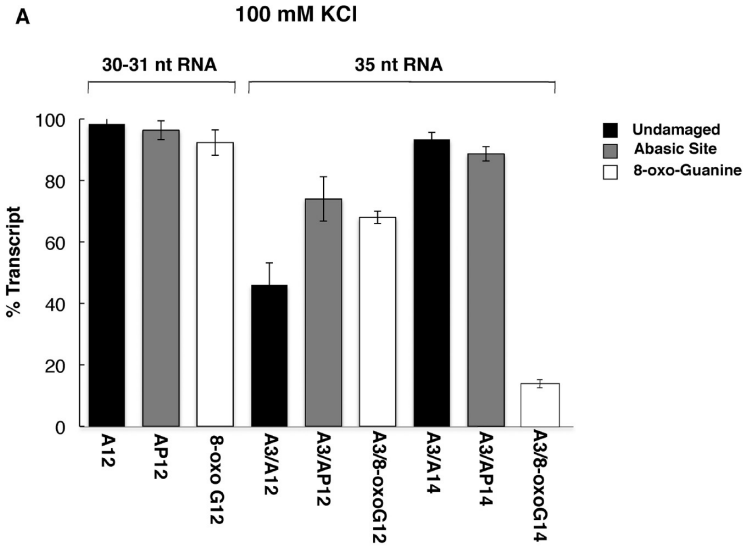
A



B

	% Transcript	
Substrate	Myc-2345 (35 nt RNA)	Myc-1245 (30-31 nt RNA)
A12	0	98 ± 2.3
AP12	0	96 ± 3.1
8-oxoG12	0	92 ± 4.2
A3/A12	46 ± 7.0	0
A3/AP12	74 ± 7.2	0
A3/8-oxoG12	68 ± 2.0	0
A3/A14	93 ± 2.3	0
A3/AP14	89 ± 2.3	0
A3/8-oxoG14	14 ± 2.0	0

Figure 6. Quantitation of the transcription data. (A) Bar graph comparing the extent of transcription arrest in each sequence analyzed. (B) Summary of the quantitation data.

substrates in the presence of 100 mM KCl, we detected a small fraction of full-length RNA and a majority of short transcripts of 30, 31, and 35 nucleotides (Figure 3B, lane 2, and Figure 3C, lane 1) as indicated by comparison with RNA markers of 30, 32, 34, and 36 nucleotides generated via transcription of shorter DNA substrates of identical sequence (Figure 3C). These shorter RNAs correspond to the arrest of transcription at T1, G2, and A6 of the c-myc repeat (Figure 2A). The 30- and 31-nucleotide RNAs are expected from T7 arrest at the myc-1245 structure and the 35-nucleotide RNAs from T7 arrest at the myc-2345 structure. This conclusion was further confirmed when transcription was conducted with oligonucleotides containing a G3 → A3 base substitution, which we have previously shown folds into myc-2345 structure (Figure 2, lane 1), or with a G12 → A12 base substitution, which we have shown folds into a myc-1245 structure (Figure 2, lane 3). Indeed, two bands of 30 and 31 nucleotides were generated when transcription was conducted after incubation of the A12 oligo in the presence of 100 mM KCl (Figure 3 C, lane 3, and Figure 4, lane 2). Under the same conditions, transcription of the A3 oligo generated one band corresponding to a 35-nucleotide RNA (Figure 3C, lane 4). Interestingly, when transcription of the wt c-myc sequence was conducted using transcription buffer provided by the manufacturer, which included 10 mM NaCl, we could detect formation of a 35-nucleotide RNA (Figure 3B, lane 3), suggesting that even a 30 min incubation in 10 mM NaCl was sufficient to promote G4 formation in the c-myc sequence. On the basis of these data, we conclude that transcription arrest is an excellent indicator of G4 DNA formation in the c-myc repeat, thus providing an alternative approach to DMS footprinting.

A Single Abasic Site or a Single 8-Oxoguanine at Position 12 of the c-myc G Repeat Promotes the Formation of myc-1245 Structure. To test the effect of two spontaneous DNA lesions on formation of G4 structure, we have substituted G12 of the c-myc repeat with a single abasic site or 8-oxoG and compared the extent of transcription arrest of the damage-containing transcription substrate with that of the wt or A12 c-myc sequence, which folds into the myc-1245 structure. We have positioned the AP site or 8-oxoG at nucleotide 12 of the c-myc repeat (Figure 4B), with the expectation that the presence of the lesion at this location would favor formation of the myc-1245 structure (Figure 4B). On the basis of the predicted myc-1245 structure, the AP site or the 8-oxoG would be located in the G11-AP/8-oxoG12-G13-G14 loop (Figure 4B). In agreement with our prediction, we found that transcription of the AP or the 8-oxoG-containing c-myc sequence preincubated in 100 mM KCl resulted in generation of 30- and 31-nucleotide transcripts, as expected from formation of the myc-1245 structure in the damage-

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containing transcription substrates (Figure 4A, lanes 4 and 6). Transcription of the lesion-containing substrates preincubated in the absence of 100 mM KCl resulted in formation of a 41-nucleotide RNA, expected from transcription arrest at the lesion, and a 74-nucleotide RNA, expected from readthrough past the lesion and generation of full-length RNA.[35−37] The extent of RNA arrest at the myc-1245 structure (>90%) in the damage-containing substrates (AP12 and 8-oxoG12) did not significantly differ from that of the A12 mutant, suggesting that the presence of the lesion in the G11−G14 DNA loop did not significantly interfere with the formation or stability of myc-1245 structure.

A G3 → A3 Base Substitution in the c-myc Repeat in Addition to an Abasic Site or an 8-OxoG at Position 12 Induces the Formation of myc-2345 Structure. We have previously shown that single-base substitutions in G repeat 1 or 3 prevent the transition either to myc-1245 (G3 → A3) or to myc-2345 structure (G12 → A12). On the basis of these findings, we predicted that the presence of both A3 and A12 base substitutions would destabilize further the formation of G4 structure in the c-myc repeat or completely prevent its formation. Surprisingly, we found that transcription of the A3/A12 mutant substrate after preincubation in the presence of 100 mM KCl generated two transcripts of 35 and 74 nucleotides, corresponding to arrest at myc-2345 structure and to full-length RNA. However, the extent of arrest of the A3/A12 double mutant (46%) (Figure 5A, lane 2, and Figure 6B) significantly decreased from that observed with the A3 single mutant (>90%) (Figure 3C, lane 4). When we substituted A12 with an AP site or an 8-oxoG, we found that transcription of the lesion-containing substrates, preincubated in the absence of 100 mM KCl, resulted in the formation of a 41-nucleotide RNA, which corresponds to transcripts arrested at the lesion (Figure 5A, lanes 3 and 5). When transcription of the lesion-containing substrates was conducted after incubation in the presence of KCl, we found that in addition to the 41-nucleotide RNA corresponding to T7 arrest at the lesion, a shorter RNA of 35 nucleotides was also synthesized (Figure 5A, lanes 4 and 6). This RNA is expected from the arrest of transcription at myc-2345 structure. The extent of arrest was ∼74% for the AP-containing substrate and ∼68% for the 8-oxoG-containing substrate, indicating that the presence of the lesion facilitated the formation of G4 structure compared to the A3/A12 double mutant (Figure 6A,B).

The Presence of a G → A Substitution at Position 3 and an 8-OxoG at Position 14 Inhibits the Formation of G4 DNA in c-myc NHEIII$_1$. To further characterize the effect of AP and 8-oxoG on the transition to G4 DNA, we have substituted G14 with an AP site or an 8-oxoG in addition to a G3 → A3 base substitution. On the basis of our DMS footprinting data on the wt and the A12 and A3 mutants, showing that G14 does not participate in G4 formation (Figures 1B and 2), we reasoned that the presence of the lesion at position 14 and of a base substitution at position 3 would not significantly affect the formation of myc-2345 structure. Indeed, when we conducted transcription on the A3/A14 double mutant, we observed >90% formation of a 35-nucleotide RNA, as expected from the formation of myc-2345 structure (Figure 5A, lane 8). This RNA was also visible, although at a much lesser extent, when transcription was conducted in the absence of KCl, suggesting that the transition to G4 is very stable (Figure 5A, lane 7). When transcription was conducted on the AP-containing substrate, we observed results similar to those for the undamaged A3/A14 double mutant (Figure 5A, lanes 9 and 10). Surprisingly, the presence of 8-oxoG at position 14 significantly decreased the level of formation of the 35-nucleotide RNA to ∼14% (Figure 5A, lane 12), indicating that 8-oxoG had a strong inhibitory effect on the formation of myc-2345 structure.

DISCUSSION

We have characterized the effect of spontaneous DNA damage on the formation of G4 DNA structure in nuclease hypersensitive element III$_1$ (NHEIII$_1$) from the c-myc gene. By DMS protection assays, we directly show that this sequence can fold into two G4 structures, myc-1245 and myc-2345 (Figures 1 and 2), in agreement with NMR and circular dichroism analyses of wt and mutated NHEIII$_1$ containing oligonucleotides.[26−29] DMS mapping data were further supported by in vitro transcription stop assays conducted with the wt and mutated NHEIII$_1$ sequences (Figure 3).[34] The sensitivity of these assays was sufficient to distinguish between c-myc-1245 and c-myc-2345 structures based on the different RNA sizes, 30−31 nucleotides compared to 35 nucleotides, of the RNAs obtained from transcription of the A12 or A3 oligonucleotides (Figure 3). Quantitation of the extent of transcription arrest revealed that both c-myc-1245 and c-myc-2345 represent a complete block to T7 RNA polymerase progression (Figure 6A,B).

On the basis of the high sensitivity of this assay and as an alternative to the use of DMS protection, because the lesions studied are sensitive to piperidine, we have utilized the T7 RNA polymerase stop assay to detect G4 DNA formation in damage-containing NHEIII$_1$.[33] First, we have positioned the AP site or the 8-oxoG at nucleotide 12 of the c-myc repeat (Figure 1), with the expectation that the lesion at this location would favor formation of myc-1245 over myc-2345 (shown in Figures 1A and 4B). On the basis of the predicted myc-1245 structure, the AP site or the 8-oxoG would be located in the G11-AP12 (or 8-oxoG12)-G13-G14 loop (Figure 4B). In agreement with our prediction, we found that the presence of either lesion at position 12 of the NHEIII$_1$ sequence resulted in transcription arrest at the first G tract (G3), as expected from formation of myc-1245 structure (Figure 4A, lanes 4 and 6). Quantitation of the extent of arrest indicated that more than 90% of the damage-containing substrates were folded in the myc-1245 structure, indicating that the presence of either lesion altered the structural transition occurring in NHEIII$_1$ by preventing formation of myc-2345.

When an additional G to A substitution was introduced at position 3 of NHEIII$_1$, which interrupts the first G run and thus is expected to interfere with formation of both myc-1245 and myc-2345 structures, we observed 35-nucleotide transcripts in addition to full-length RNAs (Figure 5A, lanes 2, 4, and 6), consistent with formation of myc-2345 structure. Interestingly, the extent of arrest of the AP- or 8-oxoG-containing substrates increased from ∼46 to ∼74% compared to that of the A3/A12 undamaged substrates, suggesting that the presence of the lesion had a positive effect on the formation of G4 structure. Our data indicate that the AP site or the 8-oxoG may be accommodated in the G4 structure, as recently shown for AP- or 8-oxoG-containing G4 structure of the human telomere DNA sequence and for the AP site-containing parallel quadruplex structure formed in the sequence d-(TGGGGdspacerT).[38−40] We cannot exclude the possibility that in the G tract of G11−G14, where G11−G13 participate in quartet formation (Figure 1, myc 2345, and Figure 2, lane 3),

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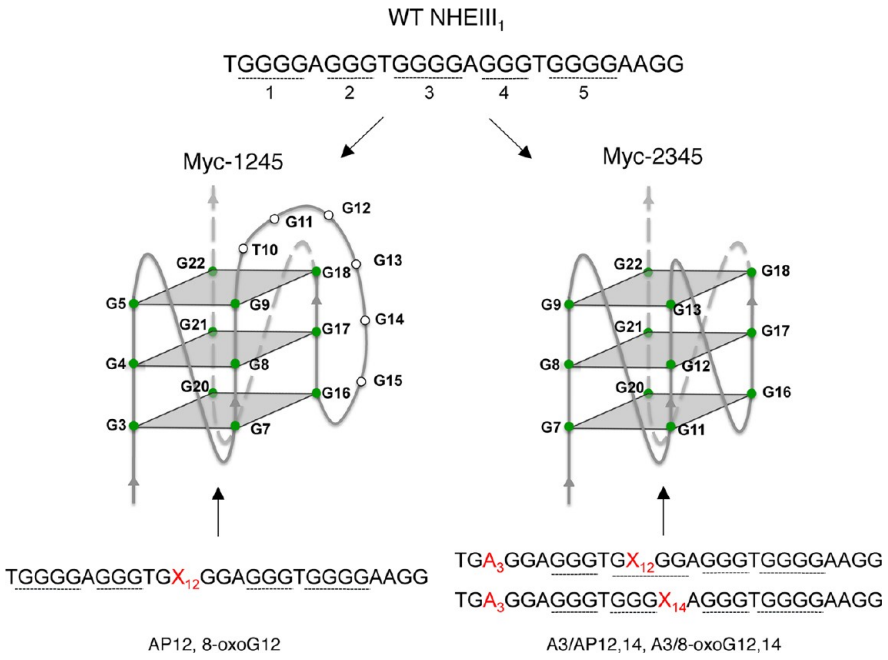

Figure 7. Summary of the effect of spontaneous DNA lesions of the AP site and 8-oxoguanine on the formation of G4 structure in c-myc $NHEIII_1$. Two G4 structures, myc-1245 and myc-2345, can form in the wt c-myc $NHEIII_1$ sequence. A single AP site or a single 8-oxoG at position 12 promotes the formation of myc-1245 structure. When a second base substitution at position 3 is introduced, which is expected to prevent myc-1245 structure, the $NHEIII_1$ sequence folds into myc-2345.

the lesion at position 12 may bulge out instead of participating in quartet formation to accommodate G13 and G14 in the middle and 3′ quartet (Figure 5B).

The presence of an AP site at position 14 in addition to a G to A base substitution at position 3 did not significantly affect the formation of myc-2345 (in Figure 5A, compare lanes 8 and 10) as predicted from substitution of a G at the 3′ end of a GGGG tract with an AP site. Surprisingly, we found that the presence of an 8-oxoG at position 14 in addition to a G to A base substitution at position 3 reduced the level of transcription arrest to ∼14% (Figure 5A, lane 12) compared to the values of ∼93 and ∼89% observed for the A3/A14 and A3/AP14 substrates, respectively (Figure 5, lanes 8 and 10), indicating that the location of the 8-oxoG (G12 vs G14) in the G run dramatically affected the formation of G4 structure. On the basis of the myc-2345 structure (Figure 1A and Figure 2, lane 3), 8-oxoG14 would be located in the loop normally formed by G14 and A15 (Figure 5B). We speculate that the steric hindrance of the 8-oxoG14 hydroxyl group might interfere with and thus perturb the geometry and stacking interactions necessary for the proper assembly of the adjacent quartets. However, on the basis of our transcription data, we cannot rule out the possibility that other G4 structures that employ the second G tract may also be formed.

Biological Implications. Our data showing that a single 8-oxoG or a single AP site alter the formation of G4 structure in $NHEIII_1$ have significant implications for the repair of spontaneous lesions in this unusual sequence context and the transcriptional regulation of this critical promoter region. Abasic sites and 8-oxoguanine are repaired by base excision repair, a ubiquitous repair pathway essential for cell survival. This excision repair process consists of sequential enzymatic reactions that lead from damage recognition to removal of the lesion and synthesis of an intact DNA strand utilizing as a template the complementary strand.[4] Our findings that the presence of an AP site or an 8-oxoG does not eliminate but instead modulates the formation of G4 structure suggest that the sequential steps of the repair reaction may likely be altered by the presence of the unusual structure, resulting in inefficient, incomplete, and/or faulty repair of the lesion and subsequent mutagenesis. For example, repair of an AP site by AP endonuclease when located in the loop of c-myc-1245 may be inefficient because of the single-stranded nature of the substrate for repair. In agreement with this prediction, it has been shown that human Ape1 cleaves DNA at AP sites less efficiently when these lesions are in single-stranded DNA compared to their double-stranded counterpart.[41,42] Similarly, repair of 8-oxoG by human OGG1 from the loop of a hairpin substrate occurs with a rate that is ∼700-fold slower than that observed for the duplex substrate.[43] In addition, the introduction of these types of DNA damage into the G4-forming structures could significantly impact how they are repaired if the G4 structures become stabilized, such as the possibility of initiating long patch base excision repair when these lesions are present in an unusual form that may preclude short patch base excision repair.

The myc-2345 structure has recently been shown to be biologically relevant in transcription regulation of the c-myc gene, based on transfection studies with plasmids containing wt and mutant $NHEIII_1$ sequences showing that destabilization of myc-2345 results in a 3-fold increase in the transcriptional activity of the c-myc promoter. Furthermore, its stabilization by a ligand weakens or suppresses c-myc transcriptional activation.[19] Our findings, showing that the presence and location of a spontaneous lesion within the $NHEIII_1$ sequence modulate not only the extent (Figure 6B) but also the type (Figure 5) of structure formed in $NHEIII_1$, suggest that the introduction of these lesions may impact transcription and/or expression from the c-myc promoter or other promoters with similar structures.

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ABBREVIATIONS

G4 DNA, G quadruplex DNA; T7 RNAP, T7 RNA polymerase; DMS, dimethyl sulfate; AP site, apurinic/apyrimidinic site; 8-oxoG, 7,8-dihydro-8-oxoguanine.

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