Stabilization of G-quadruplex in the BCL2 promoter region in double-stranded DNA by invading short PNAs

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ABSTRACT
Numerous regulatory genes have G-rich regions that can potentially form quadruplex structures, possibly playing a role in transcription regulation. We studied a G-rich sequence in the BCL2 gene 176-bp upstream of the P1 promoter for G-quadruplex formation. Using circular dichroism (CD), thermal denaturation and dimethyl sulfate (DMS) footprinting, we found that a single-stranded oligonucleotide with the sequence of the BCL2 G-rich region forms a potassium-stabilized G-quadruplex. To study G-quadruplex formation in double-stranded DNA, the G-rich sequence of the BCL2 gene was inserted into plasmid DNA. We found that a G-quadruplex did not form in the insert at physiological conditions. To induce G-quadruplex formation, we used short peptide nucleic acids (PNAs) that bind to the complementary C-rich strand. We examined both short duplex-forming PNAs, complementary to the central part of the BCL2 gene, and triplex-forming bis-PNAs, complementary to sequences adjacent to the G-rich BCL2 region. Using a DMS protection assay, we demonstrated G-quadruplex formation within the G-rich sequence from the promoter region of the human BCL2 gene in plasmid DNA. Our results show that molecules binding the complementary C-strand facilitate G-quadruplex formation and introduce a new mode of PNA-mediated sequence-specific targeting.

INTRODUCTION
The BCL2 gene, named after B-cell lymphoma 2, encodes an apoptosis inhibitor protein. BCL2 protein is localized on the mitochondrial membrane and helps maintain the delicate balance between programmed cell death and survival. Due to its role in apoptosis regulation, the BCL2 gene is an important target in anticancer treatment using pro-apoptotic drugs. Increased levels of BCL2 mRNA are found in many human cancers, such as B- and T-cell lymphomas (1), breast (2), cervical (3), lung (4) and prostate/colorectal cancers (5). Thus, the BCL2 gene is included in the proto-oncogene family as a potent factor inhibiting apoptosis (6). Furthermore, insufficient expression of the BCL2 gene may lead to increased apoptotic activity in cells resulting in various degenerative diseases such as multiple sclerosis (7), Alzheimer’s disease (8), Parkinson’s disease (9), stroke (10) and spinal cord injuries (11).

The human BCL2 gene has two promoters, P1 and P2 (12). P1, the major promoter, is located 1.3- to 1.5-kbp upstream of the translation start site (spanning from −3934 to −1280 bp). Although it has a few TATA regions, the P1 promoter is mainly GC rich, with numerous transcription initiation sites. The P2 promoter has multiple TATA regions and is located 1.3-kbp downstream of P1. Recently, another promoter (designated as M) was discovered in the region between P1 and P2 sites. Promoter M acts in a p53-dependent manner and has suppressive activity on P1 and P2 sites. Promoter M appears to act as the main driving force regulating BCL2 transcription.

Hurley et al. (14, 15) describe the formation of quadruplex structures in a GC-rich region located −58 to −19 bp upstream of the BCL2 P1 promoter (indicated on Figure 1 as bcl2G4-1). Multiple transcription factors bind to this region, which suggests its importance for P1 promoter regulation (16–18). For our study, we selected another G-rich sequence from the BCL2 gene promoter located 176-bp upstream of P1 promoter that exhibited G-quadruplex forming potential. We refer to this sequence as bcl2G4-2 (Figure 1).
Guanine-rich sequences with quadruplex forming potential are located in the promoter regions of numerous genes, especially regulatory genes (19). Quadruplex formation has been examined in several genes such as PDGF-A (20,21), VEGF (21–23), c-myc (21,24), KRAS (25), BCL2 (14,15). In most cases, G-quadruplex formation was studied in single-stranded DNA (ssDNA) oligonucleotides under various conditions in vitro. Data on G-quadruplex formation in double-stranded (ds) plasmids are rare, likely reflecting a lower potential for these structures to form under supercoiling stress conditions compared with other non-B DNA conformations such as Z-DNA or H-DNA triplex (26,27). One of the first examples of quadruplex formation in plasmids was observed by electron microscopy. The authors developed a plasmid with guanine-rich inserts and observed a 'G-loop' DNA/RNA hybrid on the C-rich strand and a G-quadruplex on the complimentary strand. They concluded that the RNA/DNA hybrid was critical for G-quadruplex stabilization in the post-transcriptional G-loops (28).

In this article, we report the formation of G-quadruplexes in ds plasmid DNA when peptide nucleic acids (PNAs) bind to the complimentary C-strand. PNAs are nucleic acid mimics with the canonical nucleobases connected to an achiral, uncharged polyamide backbone (29,30). PNA forms very stable duplexes and triplexes with complementary DNA, displacing the complementary DNA strand in DNA duplexes and producing so-called PD-loops (31–33). We designed short duplex- and triplex-forming PNAs that are able to invade dsDNA of the BCL2 gene by selectively binding to the complementary cytosine-rich sequence, thus promoting quadruplex formation in the guanine-rich segments. Because the anticipated quadruplex would be solely DNA, this approach differs from strategies targeting G-rich sequences by forming PNA/DNA hybrid quadruplexes (34–36).

Initially, we examined the G-quadruplex forming potential of a single-stranded region of the BCL2 gene (bcl2G4-2) in vitro. We then inserted the bcl2G4-2 sequence into plasmid DNA and studied whether quadruplexes could form in the G-rich strand of dsDNA in different conditions. Specifically, we employed several types of PNA oligomers and the quadruplex-stabilizing drug telomestatin. Our results show that PNA binding to the complementary C-strand is required for the G-quadruplex formation in the G-rich strand and suggest a new mode of sequence-specific targeting.

MATERIALS AND METHODS

DNA oligonucleotides

The DNA oligonucleotides (Table 1) were synthesized on an ABI394 DNA synthesizer (PE Applied Biosystems, Foster City, CA, USA), and purified by denaturing polyacrylamide gel electrophoresis (PAGE) as described previously (37). The absorbance of single-stranded oligonucleotide solutions were measured at 260 nm on an HP 8452A Diode Array Spectrophotometer, and the concentrations were calculated with extinction coefficient calculator software (http://www.basic.northwestern.edu/biotools/oligocalc.html). Several guanosines in BCL2doublePPG (marked as P) were substituted with 8-aza-7-deaza-2′-deoxyguanosine (PPG) (38). Partially duplex DNA oligonucleotides were obtained by annealing of complementary strands by slow cooling from 95°C to room temperature. The DNA oligonucleotides were incubated in 20 mM Tris–HCl buffer solution (pH 7.4) at 37°C for 1 h and in the presence or absence of 100 mM NaCl or 100 mM KCl.

PNAs

Bis-PNAs were purchased from Panagene, South Korea. Central-binding PNAs (two short G-rich sequences) were synthesized via Boc-mediated solid phase synthesis on a 433A Automated Peptide Synthesizer from Applied Biosystems (39). Boc-protected PNA monomers were purchased from PE Applied Biosystems. PNA oligomer quality was tested using an HP 1050 high performance liquid chromatography (HPLC) and spectrophoto-metrically (HP 8452A Diode Array Spectrophotometer). PNA concentration was measured at 260 nm on an HP 8452A Diode Array Spectrophotometer, and was calculated with extinction coefficient calculator software (http://www.basic.northwestern.edu/biotools/oligocalc.html). Prior to all experiments, PNAs were incubated in a shaker for 15 min at 42°C and their concentration was measured before mixing with DNA samples. PNA sequences are presented in Table 2.

Plasmids

Plasmids carrying the appropriate inserts were obtained by cloning of the BCL2single oligonucleotide (the same G-rich sequence with short TTCCTTT regions for duplex formation with bis-PNAs) with the corresponding complementary strand into pCR-Blunt vector using the Zero Blunt PCR Cloning Kit (Invitrogen, Carlsbad, CA, USA). Constructs were incorporated and amplified using the One Shot TOP10 Chemically Competent Escherichia coli (Invitrogen) and selected with 50 µg/ml kanamycin. Plasmid DNA was purified using the Plasmid Maxi Kit (Qiagen, Valencia, CA, USA). All procedures were performed according to the manufacturer’s recommendations. Plasmids were additionally purified by ultracentrifugation in a CsCl gradient (40). All plasmids were sequenced to confirm the desired insertion using the Maxam–Gilbert sequencing procedure (41). Incubation of plasmids with PNAs was performed in 20 mM potassium acetate (pH 5.2) with or without 10 µM telomestatin at 37°C overnight with molar ratio 100:1 PNA to DNA.

Dimethyl sulfate footprinting of oligonucleotides

DNA oligonucleotides were 5′–32P-labeled with [γ,32P]-ATP (Perkin Elmer, Waltham, MA, USA) by T4 polynucleotide kinase using standard protocol followed by purification on G-25 microcolumns (GE Healthcare, UK). After incubation under various conditions, samples were probed with 2 µl 10% dimethyl sulfate (DMS) in ethanol for 15 min at 15°C. The reaction was terminated

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with stop solution on ice (1.5 M sodium acetate, pH 5.2, 1 M β-mercaptoethanol, 100 μg/ml yeast tRNA) followed by ethanol precipitation and washing with 70% ethanol. After incubation in 10% piperidine at 95°C for 20 min and repeated lyophilizations, samples were analyzed by electrophoresis in 12% denaturing polyacrylamide gels. Gels were quantified using BAS-2500 Bioimager (FUJI Medical Systems USA, Stamford, CT, USA). To measure the intensity of the individual bands, the intensity profile of each lane was generated from the digitized gel image using Image Gauge software (FUJI Medical Systems USA). Band intensities were normalized with control samples (Tris–HCl buffer), inverse values are presented.

**Thermal denaturation spectrophotometry**

Absorbance spectra measurements were performed on an HP 8452A Diode Array Spectrophotometer. DNA oligonucleotides were placed in quartz cuvettes with an optical path length of 1 cm. Absorbance spectra were recorded in 220–335 nm range from 90°C to 2°C during slow cooling down of the samples (0.5°C per min) in the presence of 100 mM KCl in 20 mM Tris–HCl buffer solution (pH 7.4). Spectra at 90°C were subtracted from those at 20°C and normalized on oligonucleotide concentration. The temperature-dependant absorbance at 295 nm was also recorded.

**Circular dichroism spectroscopy**

Circular dichroism (CD) spectra measurements were performed on Jasco J-810 spectrophotometer in a 2-mm path length cuvette and a wavelength scanning speed of 100 nm/min. DNA oligonucleotides were incubated for G-quadruplex formation as described above. CD spectra of samples in the presence of 100 mM KCl and 20 mM Tris–HCl (pH 7.4) were measured at 20°C in the range from 200 nM to 320 nM. The spectra (the averages of three scans) were corrected on blank buffer spectra and normalized on oligonucleotide concentration.

**Chemical probing of plasmid DNA**

Plasmid DNA samples contained 1 μg of plasmid in 40 mM TE buffer (pH 7.4) with or without 100 mM KCl and 100 mM NaCl or 20 mM potassium acetate (pH 5.2). Plasmids incubated with PNAs were chemically modified to a total volume of 50 μl with 2.5 mM OsO₄ plus 2.5 mM 2,2′-dipyridyl disulfide for 5 min at room temperature, with 2 μl diethylpyrocarbonate (DEPC) for 5 min at room temperature, or with 2 μl 10% DMS in ethanol for 15 min at 15°C. Reactions were terminated with stop solution on ice (1.5 M sodium acetate, pH 5.2, 1 M β-mercaptoethanol and 100 μg/ml yeast tRNA) followed by ethanol precipitation and washing with 70% ethanol. Modified plasmids were cut by incubation with Fast Digest SpeI and Kpn1 restriction enzymes (Fermentas, Hanover, MD, USA) for 1 h at 37°C followed by 3′,5′-labeling with [α-32P]-dCTP (Perkin Elmer) by Klenow fragment of DNA polymerase I (Fermentas). After purification on G-50 microcolumns (GE Healthcare) followed by incubation in 10% piperidine at 95°C for 20 min and repeated lyophilizations, samples were analyzed by electrophoresis on 12% denaturing polyacrylamide gels without fragment purification, allowing the shorter fragment to run off the gel. For DMS protection analysis, intensities of bands corresponding to guanines were measured and normalized on that of guanines outside G-rich sequence.

**RESULTS**

**Formation of G-quadruplex structures by G-rich strands of BCL2 gene**

To test the possibility of G-quadruplex formation in the BCL2 guanine-rich sequence, we synthesized ssDNA with the sequences from the region of BCL2 176-bp upstream of the P1 promoter (bcl2G4-2, Figure 1). The guanine-rich region bcl2G4-2 was adjacent to a short ds portion of the molecule to better mimic conditions in a plasmid and to prevent single-strand tail interaction, possibly affecting quadruplex formation (42). The resulting BCL2double is a partially ds oligonucleotide with a single-stranded G-rich region with seven guanine runs (Table 1). For a positive control, T2G4double oligonucleotide was synthesized, which has a ds part similar to BCL2double and a single-stranded G-rich region from *Tetrahymena thermophila* telomeres. For a negative control, we synthesized an oligomer containing multiple PPG

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Promoter structure of the human BCL2 gene; shown bcl2G4-2 insert is a 51-mer sequence of guanine-rich strand. Runs of guanines are underlined and marked.
The PPG guanine analog lacks N7 nitrogen, eliminating the possibility of hydrogen bonding to the Hoogsteen face, thus precluding quadruplex formation (38). Oligonucleotide sequences are presented in Table 1. Runs of guanines are marked with roman numerals from I to VII; PPGs are marked as ‘P’.

Table 1. Sequences of DNA oligonucleotides used in the study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>BCL2 single</td>
<td>5’-TTTCTTTCCGGGAGGAGCGGGGACGGGAATCGGTTTTCCCT-3’</td>
</tr>
<tr>
<td>BCL2 double</td>
<td>5’-GAGAACGTCAACATACAGTGGTTAGGGGACGGGAATCGGTTTTCCCT-3’</td>
</tr>
<tr>
<td>BCL2 double PPG</td>
<td>5’-GAGAACGTCAACATACAGTGGTTAGGGGACGGGACCGGPPGCGAGGGPPGCGGCGCTCGGGTT-3’</td>
</tr>
<tr>
<td>T2G4 double</td>
<td>5’-CTCTGTGATGGGGACTATGTCACAAT-5’</td>
</tr>
</tbody>
</table>

P = 8-aza-7-deaz-2’-deoxyguanosine (PPG).

Figure 2. CD spectral analysis of BCL2 double (solid line) and BCL2 double PPG (dash line). CD spectra of the samples in the presence of 100 mM KCl and 20 mM Tris–HCl (pH 7.4) were measured at 20°C in the range from 200 nm to 320 nm. The spectra (the averages of three scans) were corrected on blank buffer spectrum and normalized on oligonucleotide concentration.

CD spectroscopy

CD spectroscopy studies were performed to obtain evidence for the presence of G-quadruplex structures in the BCL2 sequence. CD spectroscopy has been widely used for the characterization of G-quadruplex-forming oligonucleotides (43,44). We acquired CD spectra of BCL2 double and BCL2 double PPG in 100 mM KCl and 20 mM Tris–HCl buffer (pH 7.4) at 20°C. DNA oligonucleotides were incubated for G-quadruplex formation in the buffer solution at 37°C for 1 h. Data are shown in Figure 2. The solid line represents the CD spectrum of BCL2 double, while a dashed line represents the control, BCL2 double PPG. The BCL2 double CD spectrum has a prominent positive peak at ~260 nm and a negative peak at 240 nm. However, the CD spectrum of BCL2 double PPG is almost flat, lacking distinguishable maxima or minima. The lack of ellipticity evidenced in BCL2 double PPG can be attributed to the lack of N7 nitrogen hydrogen bonding capabilities, eliminating the possibility of quadruplex formation. CD spectra are generally affected by the orientation of guanine residues relative to the sugar moieties, so that parallel and antiparallel quadruplexes can be distinguished. Based on the spectra of short G-rich telomeric oligonucleotides that form tetramolecular parallel structures, the positive peak near 260 nm and negative peak near 240 nm are characteristic for the parallel G-quadruplex conformation (45–47). Based on these data, the BCL2 double sequence forms G-quadruplexes in the presence of potassium ions with a CD spectrum resembling that of parallel quadruplex.
Thermal denaturation

Further spectroscopic evidence for the presence of G-quadruplex structures in the BCL2 promoter region was obtained from annealing studies of BCL2 double and BCL2 doublePPG. Absorbance spectra were recorded in 220–335 nm range at 20°C and 90°C during slow cooling of the samples (0.5°C per min) in the presence of 100 mM KCl in 20 mM Tris–HCl buffer solution (pH 7.4). Figure 3A shows data for BCL2 double, and Figure 3B for BCL2 doublePPG. The renaturation curve at 295 nm reveals a transition with melting temperature (Tm) of ~24°C (Figure 3A, upper panel) for BCL2 double. No melting transition was evident for BCL2 doublePPG. Spectra at 90°C were subtracted from those at 20°C and normalized on oligonucleotide concentration. It was previously described that differential absorbance spectra of G-quadruplex structures have a characteristic peak in absorbance at 295 nm (48). This peak is observed in the case of BCL2 double along with the expected negative peak for oligonucleotides at 260 nm (Figure 3A, bottom panel). However, the differential spectrum of BCL2 doublePPG lacks the peak at 295 nm (Figure 3B, bottom panel). Thus, thermal denaturation studies support G-quadruplex formation in the BCL2 double sequence in potassium-containing buffer.

Chemical probing

For further evidence of G-quadruplex formation, we probed BCL2 double and T2G4 double with DMS (see ‘Materials and Methods’ section). This assay detects guanines in a G-quadruplex due to protection of their N7 positions from DMS modification. Samples were probed in 100 mM KCl, 20 mM Tris–HCl buffer solution (pH 7.4) and analyzed in 12% denaturing PAGE (Figure 4, right panel). The intensity of the individual bands in gels was measured and normalized to control samples probed with DMS in the same Tris–HCl buffer without KCl. The inverse values are presented on the graph (Figure 4). Both oligonucleotides tend to form G-quadruplex structures in the presence of potassium ions after incubation at 37°C for 1 h. DMS protection is observed in the range of 60–90% (quantified for BCL2 double) depending on the position of guanines along the sequence. Guanines with a higher degree of protection that apparently participate in G-quadruplex formation are marked with asterisks. They correspond to the G-runs II, III, V and VI. Incubation of BCL2 double and T2G4 double in 100 mM NaCl at 37°C for 1 h resulted in strong DMS protection of guanines in T2G4 double and very weak DMS protection in the case of BCL2 double (very close to control without NaCl). These results suggest that T2G4 double forms G-quadruplex structures in the presence of both potassium and sodium ions (Supplementary Figure S1), while BCL2 double formed quadruplexes only in the presence of potassium.

G-quadruplex formation in plasmids

After showing evidence that the G-rich sequence of the BCL2 promoter has the potential to form G-quadruplexes in single-stranded oligonucleotides, we extended our studies of these structures to ds plasmid DNA. We used several chemical probes to quantify local dsDNA melting
at the desired location. First, we probed DNA with OsO₄ and DEPC, allowing us to determine if thymines and adenines, respectively, are open within the insert. Second, we used DMS probing to reveal quadruplex formation from the protection at the N7 position. We used plasmid samples with natural superhelical density. Samples were incubated in the presence or absence of 100 mM KCl, 100 mM NaCl and 10 μM telomestatin for 1 h at 37°C. Probing with DEPC and OsO₄ did not show local melting in the insert region (Supplementary Figure S2). Overall, we were not able to detect any evidence of stable local DNA melting or G-quadruplex formation within the insert in the presence of potassium, sodium or telomestatin.

G-quadruplex formation in plasmids incubated with PNA

Because local melting conditions likely contribute to the quadruplex formation in plasmid DNA, PNA probes were designed and synthesized to selectively bind the cytosine-rich complementary strand of the guanine-rich BCL2 sequence. Two PNA designs were employed: bis-PNAs that form triplexes with DNA regions bordering the guanine-rich region and central-binding PNAs (cPNAs) that form duplexes to the complementary cytosine-rich strand directly across from the quadruplex forming region. The PNAs used are presented in Table 2, and the concept is depicted in Figure 5. Since triplex-forming PNAs require a polypurine–polypyrimidine sequence, we added TTTCCTT regions to both ends of the G-rich sequence (BCL2single, Table 1). Incubation of plasmids with or without PNAs and with or without telomestatin was performed in 20 mM potassium acetate (pH 5.2) at 37°C overnight with molar ratio 100:1 PNA to DNA. Low pH buffer was used because protonated cytosines are required for stable triplex formation. Figure 6 represents the results of probing the plasmid DNA with a BCL2single insert with OsO₄, DEPC and DMS. Only the G-rich strand was analyzed. Samples were incubated in 20 mM KOAc, pH 5.2. Probing procedures were the same as in the previous step of the experiment. Control samples were incubated only in KOAc buffer (lanes 1, 7 and 13). Experimental samples were incubated with bis-PNAs (lanes 3, 9 and 15), central-binding PNAs (lanes 2, 8 and 14), both PNA types (lanes 4, 10 and 16), both PNA types with 10 μM telomestatin (lanes 5, 11 and 17) and only 10 μM telomestatin (lanes 6, 12 and 18). Lanes 1–6, 7–12 and 13–18 show probing with OsO₄, DEPC and DMS, respectively. Results suggest stable binding of bis-PNAs and central-binding PNAs to naturally supercoiled plasmid DNA. Data in Figure 6 clearly demonstrate

Table 2. Sequences of PNA oligonucleotides used in the study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bis-PNA (openers)</td>
<td>(Lys)₂-TTJ-JTT-T-(eg₁)₃-TTT-CCT-T–NH₂</td>
</tr>
<tr>
<td>cPNA₁</td>
<td>Lys–GGCGCGAGG–NH₂</td>
</tr>
<tr>
<td>cPNA₂</td>
<td>Lys–GCGAGGGA–NH₂</td>
</tr>
</tbody>
</table>

eg₁ = 8-amino-3,6-dioxoacontic acid linker.

Figure 4. DMS footprinting of BCL2double and T2G4double. Samples were incubated in the presence or absence of 100 mM NaCl (open circles) and 100 mM KCl (closed circles) in 20 mM Tris–HCl buffer solution (pH 7.4) at 37°C for 1 h and then probed with 2 μl 10% DMS in ethanol for 15 min at 15°C. For quantification, band intensities were normalized with control samples (20 mM Tris–HCl buffer alone), and inverse values are presented on graphs to the left. Guanines marked with asterisk are the most protected. Guanine runs are marked with roman numerals (I–VII).
local melting of the targeted DNA region with opening of the G-rich strand. OsO₄ modifications of thymines in lanes 3, 4 and 5 correspond to binding of bis-PNA openers to the complementary C-rich strand, while OsO₄ modification of thymines in lanes 2, 4 and 5 correspond to binding of cPNAs to the central part of the complementary strand within the insert. Lanes 8, 10 and 11 reveal opening of adenines as a result of cPNA invasion and binding to the complementary strand of the insert. The highest yield of modification was observed with a combination of both bis-PNA openers and central-binding PNAs with and without telomestatin (lanes 4, 5, 10 and 11 in Figure 6).

Next, we tested for G-quadruplex formation using a DMS protection assay. We measured the intensity of the individual bands after DMS probing (lanes 12–18) and normalized to guanines outside the G-rich sequence. Guanine protection from DMS was observed in all experimental lanes (lanes 14–17 in Figure 6) except for the sample incubated with only telomestatin (lane 18). The panel on the left in Figure 6 represents quantified data for DMS protection assay. Incubation with telomestatin alone did not lead to DMS protection; on the contrary, guanines were modified at a slightly higher rate. Samples incubated with PNAs showed a significant protection from DMS methylation. The most effective protection appeared to be in the case of incubation with

Figure 5. Proposed design for PNA binding to G-rich DNA region.

Figure 6. PNA binding studies. Chemical probing of plasmid with BCL2 insert with PNAs. Incubation of plasmids with PNAs was performed in 20 mM potassium acetate (pH 5.2) at 37°C overnight with molar ratio 100:1 PNA to DNA. Control sample was incubated only in potassium acetate buffer (lanes 1, 7 and 13). Experimental samples were incubated with bis-PNA (lanes 3, 9 and 15), central-binding PNAs (lanes 2, 8 and 14), with both PNA types without 10μM telomestatin (lanes 4, 10 and 16), with both PNA types with telomestatin (lanes 5, 11 and 17) and only with 10μM telomestatin (lanes 6, 12 and 18). Samples containing 1μg of plasmid in 20 mM potassium acetate (pH 5.2) were chemically modified to a total volume of 50μl with 2.5 mM OsO₄ plus 2.5 mM 2,2’-dipyridyl disulfide for 5 min at room temperature (lanes 1–6), 2μl DEPC for 5 min at room temperature (lanes 7–12), 2μl 10% DMS in ethanol for 15 min at 15°C (lanes 13–18). Guanine runs are marked with roman numerals (I–VII). For DMS protection analysis, intensities of bands corresponding to guanines were measured, normalized on that of guanines outside the G-rich sequence and presented as bars in the left panel.
only cPNAs (up to 70%). Incubation with bis-PNAs led to a slight guanine modification decrease by ~30%, probably as a result of insufficient local DNA melting in the central part of the insert. Interestingly, combination of bis-PNA openers and central-binding PNAs did not result in the highest DMS protection, even with addition of telomestatin (two lower panels in Figure 6); a decrease in DMS modification was observed at the level of ~50–60%. The highest DMS protection was observed in the guanine runs II, III, V, VI and VII.

Based on these data, we believe that short PNAs binding to the complementary C-strand result in local DNA melting. This allows the G-rich strand to form G-quadruplexes in the presence of potassium ions, and this change was revealed by increased guanine N7 protection from DMS modification.

**DISCUSSION**

*BCL2* is a potent oncogene that has a key role in apoptosis. Its overexpression is correlated with an increased chance of cancer transformation, progression and resistance to anticancer treatment. Several approaches have been explored to control *BCL2* expression, such as targeting the *BCL2* gene with triplex-forming oligonucleotides (49), antisense oligonucleotides (50) and inhibiting protein–protein interactions (51). A novel concept for gene-targeted therapy is based on the hypothesis that G-rich regions within gene promoters under certain conditions can form G-quadruplexes. Quadruplex structures could interact with transcription factors that regulate expression of these genes. Sequences with repeated guanine runs, with the potential to form quadruplexes, are overrepresented in the human genome (52). Tumor suppressor genes have very low levels of this type of sequence, while proto-oncogenes have very high levels of repeated guanine runs. G-rich regions have been found and studied in promoter regions of *PDGF* (20,21), *c-myc* (21,24), *VEGF* (21–23), *KRAS* (25) and *BCL2* (14,15).

In our study, we presented evidence for G-quadruplex formation in a second guanine-rich region of the *BCL2* gene in dsDNA. We designed a partially ds oligonucleotide with a single-stranded *BCL2* sequence overhang (*BCL2double*), and showed that this segment forms G-quadruplex structures. This was confirmed via DMS protection assay, thermal denaturing studies and CD spectrometry. The hyperchromic effect we observed was relatively weak. However, it is within the same order of magnitude as described in other quadruplex studies (48). A modest hyperchromic effect corresponds to incomplete DMS protection of guanines, which can indicate highly dynamic quadruplex formation. DMS protection was observed at the level of 60–90%, which suggests that the quadruplex structures are not entirely stable or that oligomers are switching between several types of possible G-quadruplex structures. Based solely on the CD data obtained, it is difficult to confirm the type of G-quadruplex structure observed, because there are several feasible quadruplex conformations (both intra- and inter-molecular) involving different runs of guanines in *BCL2double*. However, based on CD spectrometry, it appears a parallel quadruplex conformation predominates.

Next, we examined whether the G-rich sequence from the *BCL2* promoter region is still able to form a quadruplex after being inserted into a plasmid. We probed the plasmid with DMS, OsO4 and DEPC, excised and labeled the G-rich strand and studied the breaks resulting from chemical modifications of the bases. The idea was to observe local DNA melting within the insert region as a result of G-quadruplex formation. KCl and telomestatin are known quadruplex stabilizing agents, but there was no sign of DNA melting within the insert; DNA remained double stranded (data shown in Supplementary Data). We inferred that the free energy provided by natural supercoiling of the plasmid was not enough to stabilize the G-quadruplex in the G-rich strand while the C-strand remains open. If G-quadruplexes do play a role in gene expression regulation in living cells, proteins that stabilize guanine quadruplexes likely bind the complementary C-rich strand selectively or in some other way interact with transcription machinery (24). For example, Duquette et al. (28) observed quadruplex formation during transcription. They suggested that a RNA/DNA hybrid in the C-rich strand was responsible for the guanine-rich strand release from duplex and the quadruplex formation. However, it remains unclear whether quadruplex formation actually played any role in transcription regulation or if its formation was a ‘side-effect’ resulting from the peculiar properties of this model. Future studies should concentrate on interactions between nuclear proteins and quadruplex forming DNA sequences to clarify the biological role of G-quadruplexes in living cells.

We sought to examine guanine quadruplex formation and stabilization utilizing short PNAs. PNAs are nucleic acid mimics in which the phosphate–sugar backbone has been replaced by N-(2-aminoethyl)glycine linkages (29). PNAs are resistant to degradation from proteases and nucleases and rarely interact with cellular proteins. Because PNA is uncharged, it binds to complementary DNA and RNA with higher binding affinity and selectivity than natural nucleic acids. These properties make PNA a promising tool for sequence-specific gene targeting. Another remarkable feature of PNAs is their ability to invade dsDNA, forming so-called PD loops. This phenomena was first described by Nielsen et al. (29,53). Later, a mechanism of invasion was proposed (54) where a triplex between one PNA and two DNA strands is formed, followed by invasion and local DNA melting. Other experiments showed higher rates of strand invasion if triplex-forming bis-homopyrimidine PNAs were used (55,56). Interestingly, PNAs invade at higher rates to targets in DNA where the displaced DNA strand can form some sort of secondary structure (cruciform) (32).

We proposed a PNA binding design (Figure 5) implementing bis-PNAs and ‘central-binding PNAs’ (Table 2). Our experiments show consistent PNA binding, as evidenced by the release of G-rich strands from the DNA duplex based on OsO4 and DEPC-probing results.
(Figure 6). It was shown that even with only bis-PNA openers, one can observe a noticeable degree of DNA melting in the central part of the insert. Thus, PNAs were able to bind the C-rich strand of the BCL2 insert and release the G-rich strand. Furthermore, we observed quadruplex formation in the G-rich strand based on results from DMS protection studies. The highest rate (up to 70%) of G-quadruplex formation was observed with only central-binding PNAs. The lowest rate was ~30% with only bis-PNAs. As we have shown previously (56), binding of two bis-PNA openers did not result in the melting of the duplex region separating them. Surprisingly, the combination of central-binding PNAs and bis-PNAs led to protection from DMS at the level of 50–60%, below the level of protection afforded by cPNAs alone. We expected the combination of both types of PNA to produce the highest rates of quadruplex formation due to the greater length of the guanine-rich strand in single-stranded state. It is likely that other factors affect the ability of the released G-rich strand to form quadruplexes, such as the flexibility of the PNA/DNA complex with the C-rich strand. By analyzing DMS protection data, we cannot definitely determine which guanines within the BCL2 segment contribute to G-quadruplex formation. Presumably, within the studied BCL2 guanine-rich promoter sequence, there are several G-quadruplex formations involving various runs of guanines.

Recently, several groups proposed the use of G-rich quadruplex-forming PNA to target G-quadruplexes directly (34–36,57–59). In this approach, G3 runs of PNA are involved in quadruplex formation with the G-rich strand of DNA. However, if there are proteins that specifically recognize DNA G-quadruplexes for activation or inhibition of transcription, their binding to the PNA/DNA hybrid G-quadruplex is uncertain. We designed our central-binding PNA specifically to minimize G-quadruplex formation. Although we cannot completely exclude the possibility of the quadruplex formation between central-binding PNA and the G-strand in our experiment, our goal was to extrude a native G-quadruplex in the G-rich region of the promoter to subsequently study its effect on the expression of the BCL2 gene.

In conclusion, we induce G-quadruplex formation in the guanine-rich promoter region of the human BCL2 gene by C-strand-invading PNAs. Our results demonstrate a new mode of sequence-specific gene targeting using short, duplex- and triplex-forming PNAs. This approach could provide a basis for future applications of gene expression regulation through G-quadruplex stabilization. In future studies, we plan to implement these PNAs for in vivo studies concerning regulation of the BCL2 promoter through G-quadruplex stabilization.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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REFERENCES


