

# Facilitation of a structural transition in the polypurine/polypyrimidine tract within the proximal promoter region of the human VEGF gene by the presence of potassium and G-quadruplex-interactive agents

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## ABSTRACT

The proximal promoter region of the human vascular endothelial growth factor (VEGF) gene contains a polypurine/polypyrimidine tract that serves as a multiple binding site for Sp1 and Egr-1 transcription factors. This tract contains a guanine-rich sequence consisting of four runs of three or more contiguous guanines separated by one or more bases, corresponding to a general motif for the formation of an intramolecular G-quadruplex. In this study, we observed the progressive unwinding of the oligomer duplex DNA containing this region into single-stranded forms in the presence of KCl and the G-quadruplex-interactive agents TMPyP4 and telomestatin, suggesting the dynamic nature of this tract under conditions which favor the formation of the G-quadruplex structures. Subsequent footprinting studies with DNase I and S1 nucleases using a supercoiled plasmid DNA containing the human VEGF promoter region also revealed a long protected region, including the guanine-rich sequences, in the presence of KCl and telomestatin. Significantly, a striking hypersensitivity to both nucleases was observed at the 3'-side residue of the predicted G-quadruplex-forming region in the presence of KCl and telomestatin, indicating altered conformation of the human VEGF proximal promoter region surrounding the guanine-rich sequence. In contrast, when specific point mutations were introduced into specific guanine residues within the G-quadruplex-forming

region (Sp1 binding sites) to abolish G-quadruplex-forming ability, the reactivity of both nucleases toward the mutated human VEGF proximal promoter region was almost identical, even in the presence of telomestatin with KCl. This comparison of wild-type and mutant sequences strongly suggests that the formation of highly organized secondary structures such as G-quadruplexes within the G-rich region of the human VEGF promoter region is responsible for observed changes in the reactivity of both nucleases within the polypurine/polypyrimidine tract of the human VEGF gene. The formation of the G-quadruplex structures from this G-rich sequence in the human VEGF promoter is further confirmed by the CD experiments. Collectively, our results provide strong evidence that specific G-quadruplex structures can naturally be formed by the G-rich sequence within the polypurine/polypyrimidine tract of the human VEGF promoter region, raising the possibility that the transcriptional control of the VEGF gene can be modulated by G-quadruplex-interactive agents.

## INTRODUCTION

The size of most primary solid tumors is known to be limited to within ~1–2 mm in diameter because the cancer cells initially rely on pre-existing host blood vessels for a supply of oxygen and nutrients (1). However, starving tumor cells can become angiogenic by gaining the ability to direct the formation of new blood vessels for their survival (2,3). The angiogenic

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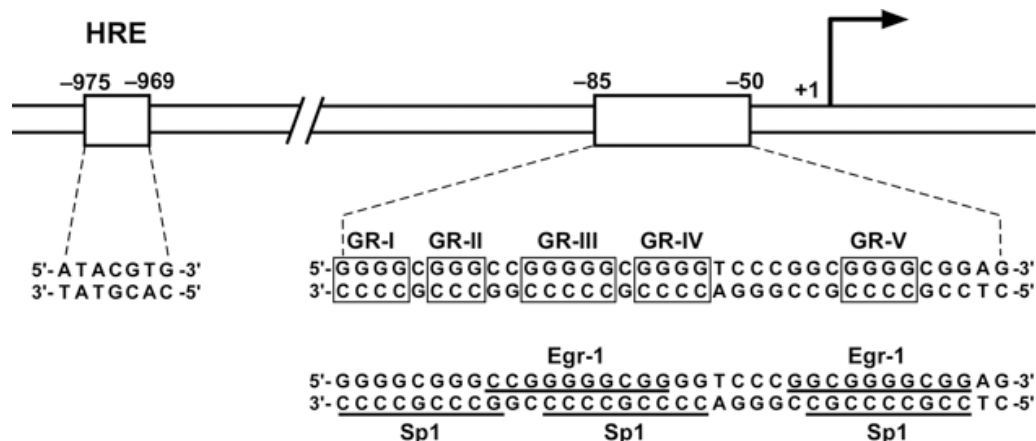
switch in cancer cells is often initiated by increased expression of vascular endothelial growth factor (VEGF), a pluripotent cytokine and angiogenic growth factor (4). VEGF consists of two identical subunits and binds to VEGF receptors on the surfaces of endothelial cells (4). The interaction of VEGF and its cognate receptors stimulates the proliferation, migration, survival and permeability of endothelial cells, resulting in the formation of new blood vessels (5). The formation of new blood vessels adjacent to primary tumor sites promotes the growth of cancer cells by providing oxygen and nutrients. In addition, newly formed blood vessels serve as escape routes for disseminating tumor cells, contributing to tumor metastasis even in locations distant from the primary tumor site (5).

The expression of human VEGF, which is frequently elevated in many types of cancer, is mainly regulated at the transcriptional level (6,7). VEGF expression is induced by a variety of factors, including hypoxia, pH, activated oncogenes, inactivated tumor suppressor genes and growth factors (8–15). The molecular basis of VEGF gene expression has been extensively studied by characterizing the *cis*-acting elements and transcription factors involved in constitutive VEGF expression in human cancer cells (9–14). The human VEGF promoter region contains putative binding sites for several transcription factors, such as HIF-1, AP-1, AP-2, Egr-1, Sp1 and many others, suggesting that they may be involved in VEGF transcriptional regulation (13,14). Functional analysis of the human VEGF promoter using the full-length VEGF promoter reporter revealed that the proximal 36 bp region (–85 to –50 relative to transcription initiation site) is essential for basal or inducible VEGF promoter activity in several human cancer cells (13,14). This region contains at least three Sp1 binding sites, each consisting of a polypurine/polypyrimidine tract (Figure 1). Although there are binding sites for other factors, such as two Egr-1 elements and one AP-2 element, all Sp1 binding sites were found to be functionally significant in constitutive VEGF promoter activity (13,14). Polypurine/polypyrimidine tracts are also found in the promoter region of other growth-related genes, including Hmga-2, EGF-R, c-Myc, I-R, AR, c-Src, c-Ki-Ras, TGF- $\beta$ , PDGF A-chain, insulin and muscle-specific protein (16–25). These sequences have been proposed to be very dynamic in

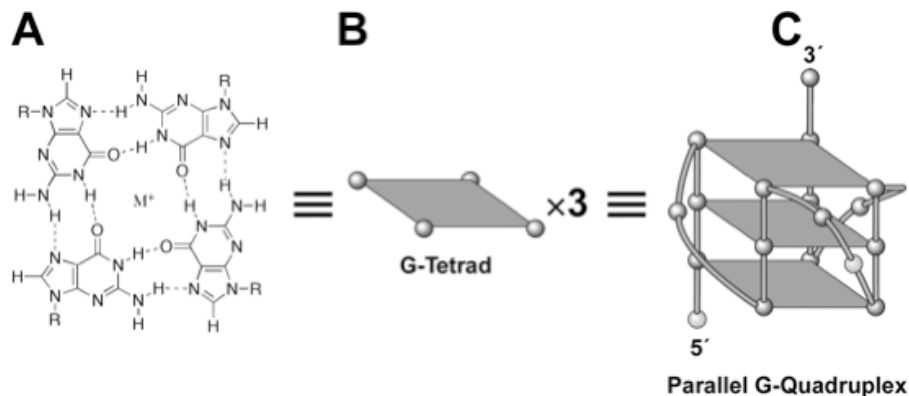
their conformation, easily adopting non-B-DNA conformations, such as melted DNA, hairpin structures, slipped helices, or others, under physiological conditions (16–25). These sequences also provide landing sites for a transcription machinery complex to initiate transcriptional activation of these genes (26,27). In particular, the guanine-rich sequences found in the polypurine/polypyrimidine tracts are known to form G-quadruplex structures consisting of two or more G-tetrads in the presence of monovalent cations such as Na<sup>+</sup> and K<sup>+</sup> under physiological conditions as shown in Figure 2A (28,29). Recently, both NMR and X-ray crystallography studies have determined the structures of various forms of G-quadruplexes, and these studies have revealed that folding patterns of these G-quadruplexes depend on the precise arrangement and composition of G-repeats, as well as on the size of the intervening sequences (30–36). Significantly, the polypurine tract of the VEGF promoter consists of five runs of at least three contiguous guanines (GR-I to GR-V in Figure 1) separated by one or more bases, conforming to the general motif capable of forming G-quadruplex structures. Therefore, we hypothesized that the polypurine/polypyrimidine tract of the VEGF promoter region is very dynamic in its structure and can easily adopt a non-B-DNA conformation under physiological conditions. Moreover, we predicted that the G-rich strand of this tract could form specific G-quadruplex structures in the presence of K<sup>+</sup> or G-quadruplex-interactive agents.

To test our hypothesis, we initially used a DNA breathing assay with an oligomer duplex containing the polypurine/polypyrimidine tract in the VEGF promoter. We anticipated that the unwinding of this oligomer duplex could be facilitated in the presence of G-quadruplex-interactive agents by trapping out and stabilizing the G-quadruplex transiently formed by the G-rich strand of the polypurine/polypyrimidine tract. In addition, we used DNase I and S1 nuclease footprinting to study the structural transition within this tract of the VEGF promoter, since the activity of these enzymes is modulated by DNA conformation (16–18).

In this article, we demonstrate that G-quadruplex-interactive agents promote the unwinding of the oligomer duplex containing the polypurine/polypyrimidine tract in the presence of K<sup>+</sup>. Footprinting experiments with DNase I and S1



**Figure 1.** Polypurine/polypyrimidine sequence located upstream (–89 to –43) of the promoter region of the VEGF gene. Runs of guanines (GR-I through GR-V) are boxed. Binding sites of the transcriptional factors Egr-1 and Sp1 are underlined.



**Figure 2.** (A) H-bonding pattern in a G-tetrad, (B) schematic diagram of the G-tetrad and (C) cartoon of an 18mer parallel G-quadruplex representing that found in the c-MYC promoter region.

nuclease using a supercoiled plasmid containing either the wild-type or mutant VEGF promoters revealed that the emergence of DNase I and S1 nuclease cleavage in the vicinity of the polypurine/polypyrimidine tract was dependent on both the capability of the wild-type sequence to form G-quadruplex structures and the presence of  $K^+$  and G-quadruplex-interactive compounds. Collectively, these results support the conclusion that G-quadruplex structures form within this polypurine/pyrimidine tract of the VEGF promoter.

## MATERIALS AND METHODS

### Drugs, enzymes and oligonucleotide DNA

TMPyP4 was purchased from Midwest Chemicals, and telomestatin was kindly provided by Dr Kazuo Shin-ya (University of Tokyo, Japan). Both drug molecules were dissolved in dimethyl sulfoxide, unless otherwise specified. T4 nucleotide kinase and *Taq* DNA polymerase were purchased from Promega. PAGE-purified oligonucleotides were obtained from Sigma Genosys.

### Polymerase stop assay

The primer DNA (P28) was 5' end-labeled using T4 polynucleotide kinase with  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  (3000 Ci/mmol, Perkin Elmer) for 1 h at 37°C and purified on a Bio-Spin 6 chromatography column (BioRad) after inactivation of the kinase by heating at 95°C for 3 min. Labeled primers were annealed to the template DNA and the primer-annealed template DNA was separated from excess labeled primer or remaining template DNA using non-denaturing 8% PAGE. The resulting primer-annealed template DNA was gel-purified and used as a substrate in a primer extension assay by *Taq* DNA polymerase, as described previously (37).

### DNA breathing assay

We designed a 59 bp oligo duplex DNA (59WT) containing the Sp1 binding sites (−101 to −43, relative to transcriptional start site) of the VEGF promoter region (see Figure 3A) in order to study the dynamic features of this promoter region. For the DNA breathing assay, the top strand of 59WT DNA was 5' end-labeled with  $^{32}\text{P}$  and incubated with increasing concentrations of TMPyP4 or telomestatin in 150 mM

KCl/TE buffer for 10 h at 37°C. The reaction also contained an 8-fold molar excess of unlabeled top (G-rich) strand as a trap to prevent the released (labeled with  $^{32}\text{P}$ ) top strands from reannealing, and the DNA was electrophoresed on an 8% native polyacrylamide gel to separate other species of DNA from the duplex form of DNA.

### Plasmid DNA

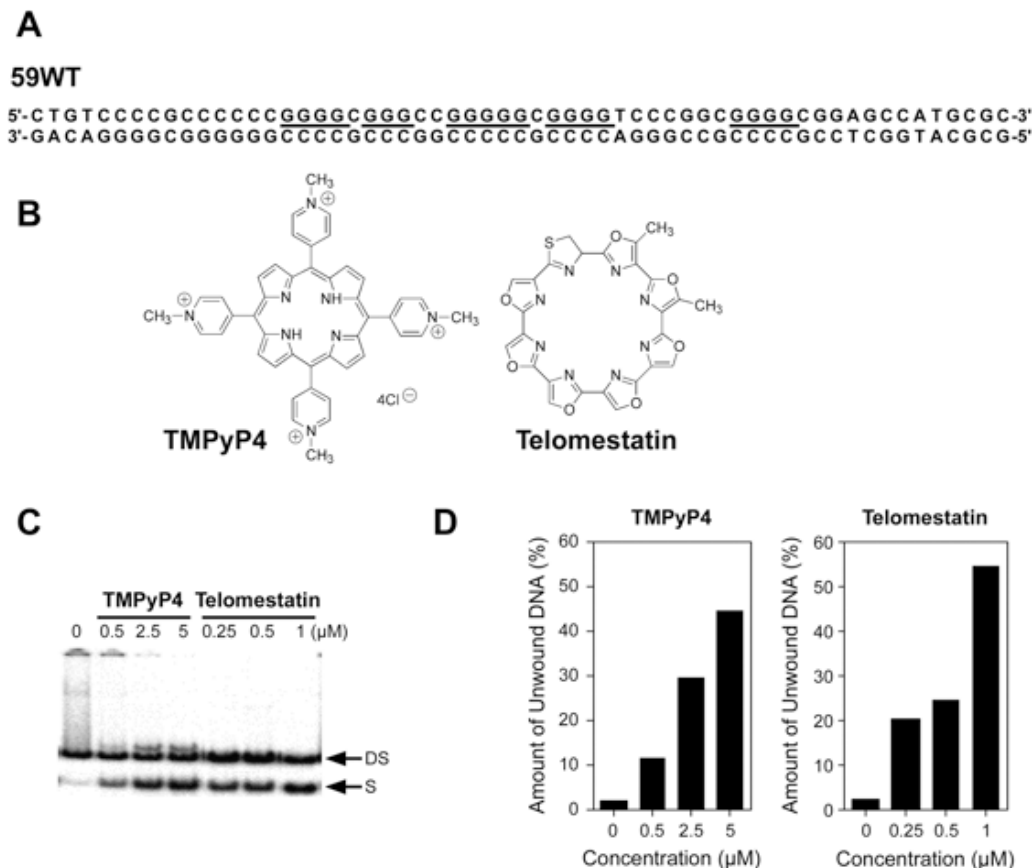
For *in vitro* footprinting of the VEGF promoter region, we used the supercoiled form of the luciferase reporter plasmid pGL3-VEGFP, which was constructed by ligating an 837 bp VEGF promoter region (−787 to +50 relative to the transcription initiation site) into the KpnI and NheI sites of pGL3-basic basic vector (Promega, Madison, WI), as described previously (14). This region of the VEGF promoter was found to support the transcription of luciferase expression in human cancer cell lines (14). A mutant reporter plasmid, pGL3-VEGFM17, was constructed by introducing point mutations into specific guanine residues within the Sp1 binding sites of the VEGF promoter regions of pGL3-VEGFP using a QuickChange mutagenesis kit (Stratagene) to destabilize or abolish the G-quadruplex-forming ability of this region.

### *In vitro* footprinting of the VEGF promoter region with DNase I and S1 nuclease

A supercoiled form of each of the plasmids (pGL3-VEGFP and pGL3-VEGFM17) was incubated in the absence of any salt, or in the presence of 100 mM KCl with and without 1  $\mu\text{M}$  telomestatin as a selective G-quadruplex-interactive agent at 37°C for 1 h and then treated with DNase I or S1 nuclease for 2 min. DNA was precipitated with ethanol and resuspended in double-distilled water after vacuum drying. To map DNase I and S1 nuclease cleavage sites on the plasmid DNA, linear amplification by PCR was performed using Thermo Sequenase Cycle Sequencing kit (USB) with  $^{32}\text{P}$ -labeled gene-specific primers d(CCCAGCGCCACGACCTCCGAGC-TACC) and d(CTGTGAACCTTGGTGGGGGTCGAGC) to amplify the top and bottom strands of the plasmid DNA, respectively.

### Circular dichroism spectroscopy

Circular dichroism (CD) spectra were recorded on a Jasco-810 spectropolarimeter (Jasco, Easton, MD), using a quartz cell of



**Figure 3.** The effect of increasing TMPyP4 and telomestatin concentrations on the conversion of 59mer duplex DNA (59WT) to alternative secondary structures. (A) Nucleotide sequence of 59WT. (B) Structures of TMPyP4 and telomestatin. (C) Autoradiogram of DNA breathing assay. Duplex DNA was titrated with TMPyP4 or telomestatin in 100 mM KCl/TE buffer at 37 or 42°C. Two bands corresponding to Watson–Crick duplex (DS) and single-stranded DNA (S) were identified, along with an isomeric form. (D) Graphical representation of data described in (C).

1 mm optical path length, an instrument scanning speed of 100 nm/min, with a response time of 1 s and over a wavelength range of 200–330 or 200–600 nm for the titration experiments with drugs. All DNA samples were dissolved in Tris–HCl buffer (20 mM, pH 7.6) to a strand concentration of 5  $\mu\text{M}$ , and where appropriate, the samples also contained 100 mM KCl. The CD spectra herein are representations of four averaged scans taken at 25°C and are baseline corrected for signal contributions due to the buffer.

## RESULTS

### Transition of duplex to a non-B-DNA conformation within the polypurine/polypyrimidine tract of the VEGF promoter region in the presence of TMPyP4 and telomestatin

In order to test if the polypurine/polypyrimidine tract of the VEGF promoter region is structurally dynamic and can easily adopt a non-B-DNA conformation under physiological conditions, a DNA breathing assay was carried out with a 59 bp oligomer duplex (59WT) containing the polypurine/polypyrimidine tract (Figure 3A). It was anticipated that the unwinding of the 59WT would occur if Watson–Crick

base pairs are destabilized by the formation of non-B-DNA structures in the Sp1 binding sites of the VEGF promoter region. In the DNA breathing assay, the oligomer duplex (59WT) labeled at the 5' end of the G-rich strand with  $^{32}\text{P}$  was incubated with increasing concentrations of the G-quadruplex-interactive agents TMPyP4 (38) or telomestatin (39) (Figure 3B) at 37°C in the presence of 150 mM KCl and then electrophoresed on an 8% native polyacrylamide gel to separate other species of DNA from the duplex form of DNA. G-quadruplex-interactive agents were included to determine if these agents promote the unwinding process by further stabilizing and trapping out the G-quadruplex structures formed by the G-rich strand. As shown in Figure 3C and D, the formation of a single-stranded DNA species was progressively increased as the concentrations of TMPyP4 and telomestatin were increased. TMPyP4 and telomestatin will prevent the conversion back to a B-DNA structure by trapping out and stabilizing the G-quadruplex structures formed in this region of the VEGF promoter. The results from the DNA unwinding study support the idea that the polypurine/polypyrimidine tract of the VEGF promoter region is very dynamic in its structure, and G-quadruplex structures can be formed by the G-rich strand of this tract under physiological conditions of potassium, pH and temperature with the addition of G-quadruplex-stabilizing agents.

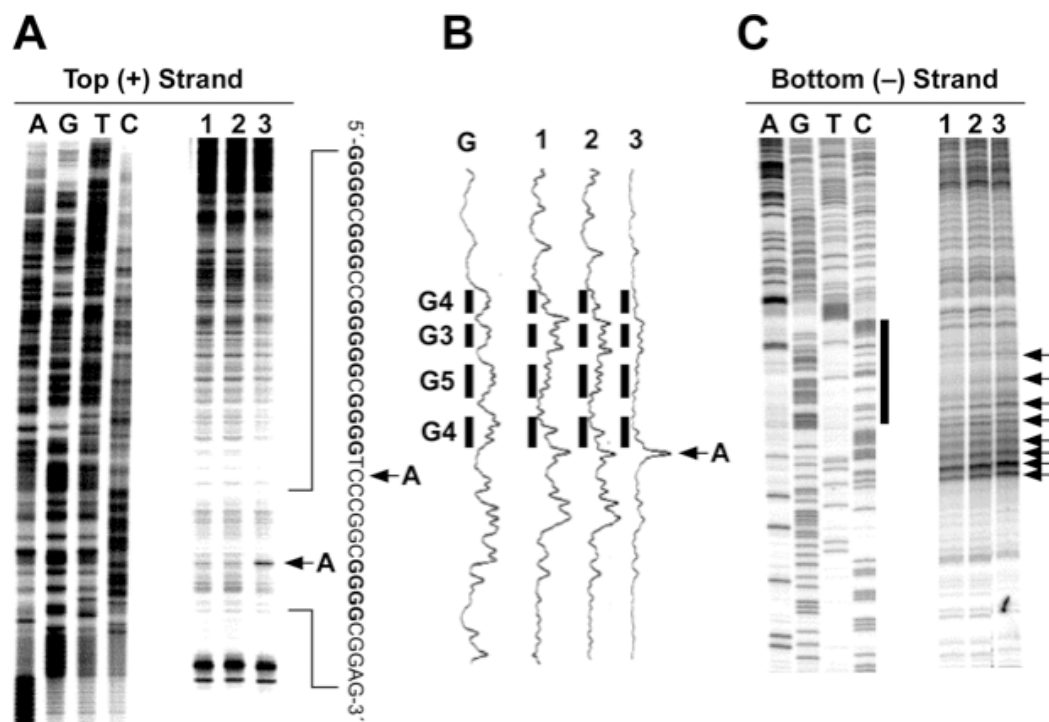


**S1 nuclease cleavage.** Since reduced reactivity to DNase I can be caused by either the presence of a single-stranded region or a highly organized secondary structure, such as G-quadruplex structures within the polypurine/polypyrimidine tract of the VEGF promoter, an additional footprinting experiment using S1 nuclease was carried out to obtain further detailed information about the conformational transition occurring in this region. As shown in Figure 5A and B, the reactivity of S1 nuclease on the top strand of the polypurine/polypyrimidine tract of the VEGF promoter was markedly reduced in the presence of KCl with telomestatin. The reduced reactivity of the VEGF promoter region surrounding the G-quadruplex-forming region to both DNase I and S1 nuclease suggests the formation of highly organized secondary structures, such as G-quadruplex structures, rather than the formation of a single-stranded form in the G-rich region of the VEGF promoter in the presence of KCl with telomestatin. Significantly, the hypersensitivity site observed with S1 nuclease also corresponds to that obtained with DNase I in the presence of KCl with telomestatin. The presence of this common hypersensitivity site to both nucleases at the 3'-side of the G-quadruplex-forming region is a strong indicator of the enhanced exposure of this particular residue to both nucleases, suggesting the presence of a junction site between the B-type DNA structure and the G-quadruplex at this site in the promoter region of the VEGF gene. In contrast, the reactivity to S1 nuclease of the opposite strand of the same region was moderately enhanced, with the hypersensitive region

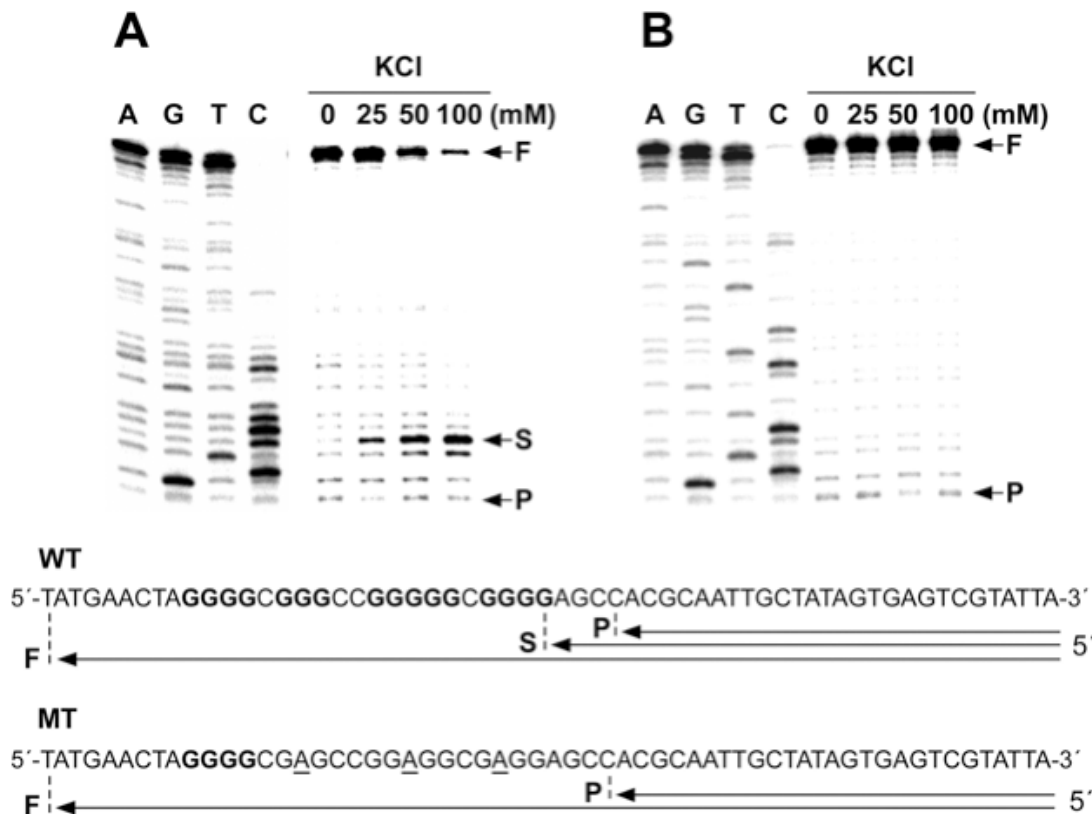
extending upstream of the G-rich tract (see arrows in Figure 5C). These results suggest that the opposite strand of the G-rich sequence and the surrounding region of the VEGF promoter exists in a single-stranded form as a result of the transition of B-type DNA structure to the G-quadruplexes under the conditions that favor such transitions.

#### Comparison of the abilities of the G-rich strands of the wild-type versus mutant VEGF promoter sequences to form stable G-quadruplex structures

In order to demonstrate the G-quadruplex-forming ability of the G-rich strand of the polypurine/polypyrimidine tract in the presence of KCl, a DNA polymerase stop assay was used, as previously described (37). This assay provides a simple and rapid way to identify DNA secondary structures, such as G-quadruplexes, *in vitro*, based on the principle that DNA polymerase is incapable of traversing these structures (37,38). Thus, the DNA polymerase, traversing toward the 5' end of the template and unable to efficiently resolve quadruplex DNA, pauses or stops 3' to the first guanine involved in a stable G-quadruplex. When a DNA template (WT) containing the G-quadruplex-forming region [d(G<sub>4</sub>CG<sub>3</sub>C<sub>2</sub>G<sub>5</sub>CG<sub>4</sub>)] was annealed with <sup>32</sup>P-labeled primers and incubated with *Taq* DNA polymerase, a potassium-dependent pause of the DNA polymerase extension was observed with this DNA template (Figure 6A). In contrast, in the absence of KCl, the DNA polymerase bypasses the G-repeat region. The KCl-dependent



**Figure 5.** *In vitro* footprinting of the VEGF promoter region with S1 nuclease. (A) Autoradiograms showing S1 nuclease cleavage sites on the top strand of a supercoiled pGL3-VEGF plasmid. Arrow A indicates the hypersensitive cleavage sites to S1 nuclease. (B) Densitometric scanning of the autoradiogram in (A). The plasmid DNA was incubated in the absence of salt (lane 1) or in the presence of 100 mM KCl without (lane 2) and with (lane 3) 1  $\mu$ M telomestatin at 37°C for 1 h before digesting with S1 nuclease. S1 nuclease cleavage sites were mapped using linear amplification by PCR with <sup>32</sup>P-labeled gene-specific primers on plasmid DNA pretreated with S1 nuclease. Arrow A indicates the hypersensitive cleavage sites to S1 nuclease. (C) Autoradiograms showing S1 cleavage sites on the bottom strand of a supercoiled pGL3-VEGF plasmid. The designation of lanes 1–3 was as in (A) above. The vertical bar next to the gel indicates the polypyrimidine tract and the arrows indicate the S1 nuclease hypersensitivity sites.



**Figure 6.** DNA polymerase stop assay to determine the ability of the VEGF promoter to form G-quadruplex structures. (A and B) The wild-type (WT) and mutant (MT) template sequences (shown below the gels) with increasing concentrations of  $K^+$  (0–100 mM). Arrows indicate the positions of the full-length product (F) of DNA synthesis, the G-quadruplex pause site (S), and the free primer (P). Lanes A, G, T and C represent dideoxy sequencing reactions with the same template as a size marker for the precise arrest sites.

pausing is observed immediately 3' to the fourth guanine run (GR-IV), presumably through stabilization of the intramolecular G-quadruplex structure, confirming that this region is indeed capable of forming intramolecular G-quadruplex structures (Figure 6A). A potassium-dependent pausing of DNA polymerase extension was not observed in the mutant DNA template (MT), in which mutations were introduced into the G-quadruplex-forming region to abolish the formation of G-quadruplex structures (Figure 6B).

#### ***In vitro* footprinting of the mutant VEGF promoter region with DNase I and S1 nuclease**

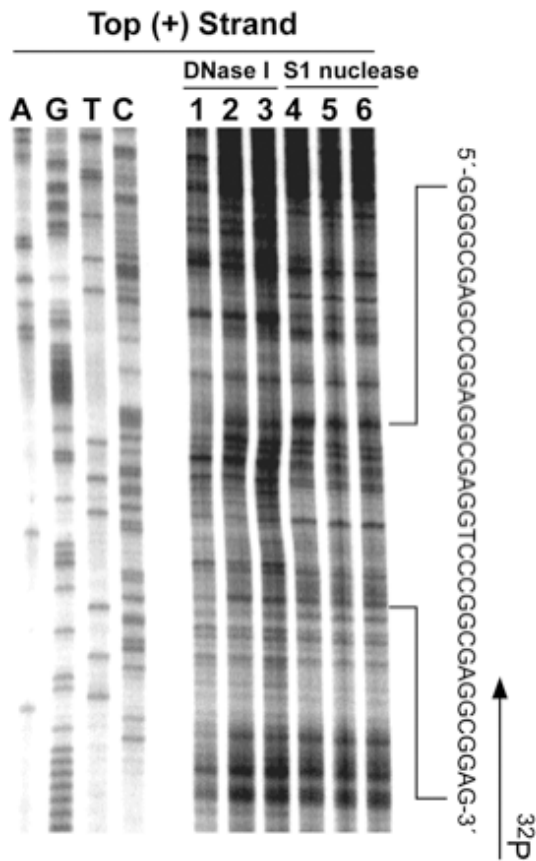
The importance of the G-quadruplex-forming ability of the polypurine/polypyrimidine tract in causing the change in the reactivity of both nucleases in the presence of KCl and telomestatin was determined using the mutant reporter plasmid pGL3-VEGFM17. This plasmid contains the same point mutations in the specific guanine residues within the G-quadruplex-forming region of the VEGF promoter as those shown in Figure 6B to destabilize or abolish the G-quadruplex-forming ability of this region. For *in vitro* footprinting, the supercoiled form of the mutant plasmid pGL3-VEGFM17 was incubated under the same conditions and with the same enzymes used in the previous experiments shown in Figures 4 and 5. As shown in Figure 7, the reactivity of both nucleases toward the mutated VEGF proximal promoter region was little changed in the presence of telomestatin with KCl,

and the hypersensitivity site was not observed with both nucleases in the presence of telomestatin with KCl. This result provides strong support for the idea that the formation of highly organized secondary structures, such as G-quadruplex structures, within the G-rich region of the VEGF promoter region is responsible for observed changes in the DNase I and S1 cleavage reactivity of the polypurine/polypyrimidine tract in the human VEGF promoter sequence.

#### **CD spectra of the G-rich strand in the VEGF promoter reveals a parallel-stranded G-quadruplex**

CD spectroscopy has been widely used to infer the presence of G-quadruplexes and is particularly useful for differentiating parallel and antiparallel G-quadruplexes (40,41). All guanines in the structure of a parallel quadruplex have an *anti*-conformation of the glycosyl bonds, resulting in a positive band at  $\sim 265$  nm and a small negative band at  $\sim 245$  nm in CD spectrum (40,41). In contrast, the guanines in an antiparallel quadruplex have alternating *syn* and *anti* glycosyl conformations along each strand, exhibiting a positive band at  $\sim 295$  nm that is associated with an antiparallel strand arrangement. The oligonucleotide used in CD studies was VEGF-PuT, d(T<sub>5</sub>G<sub>3</sub>CG<sub>3</sub>C<sub>2</sub>G<sub>5</sub>CG<sub>3</sub>T<sub>5</sub>), which contains a potential G-quadruplex forming region [d(G<sub>3</sub>CG<sub>3</sub>C<sub>2</sub>G<sub>5</sub>CG<sub>3</sub>)] of the G-rich strand in the VEGF promoter. As shown Figure 8, the CD spectra of this sequence displays the characteristic signature for the parallel-type of G-quadruplex in the presence

of increasing concentration of KCl, exhibiting a positive band at  $\sim 265$  nm and a small negative band at  $\sim 245$  nm.

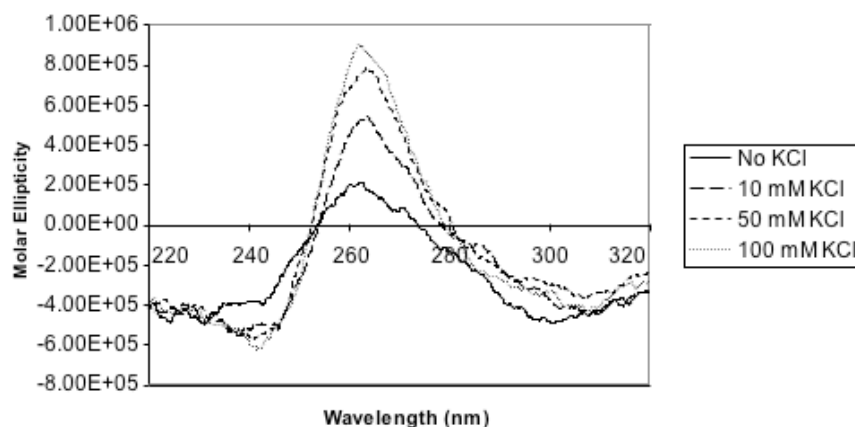


**Figure 7.** *In vitro* footprinting of the mutant VEGF promoter region with DNase I and S1 nuclease. Autoradiograms showing DNase I (lanes 1–3) and S1 (lanes 4–6) cleavage sites on the top strand of a supercoiled pGL3-VEGFM17 plasmid. This plasmid was incubated in the absence of salt (lanes 1 and 4) or in the presence of 100 mM KCl without (lanes 2 and 5) and with (lanes 3 and 6) 1  $\mu$ M telomestatin at 37°C for 1 h before digesting with nucleases. Nuclease cleavage sites were mapped using linear amplification by PCR with  $^{32}$ P-labeled gene-specific primers on mutant plasmid DNA pretreated with S1 nuclease or DNase I.

## DISCUSSION

The proposed transition of B-DNA into non-B-DNA structures, such as melted DNA, hairpin structures, slipped helices or others, has been reported in a number of growth-related mammalian genes, including Hmga2, EGF-R, c-Myc, malic enzyme, I-R, AR, c-Src, c-Ki-Ras, TGF- $\beta$ , sMtCK,  $\alpha$ 7 integrin and PDGF A-chain (16–25). Significantly, the polypurine/polypyrimidine tracts are often found in the proximal promoter region of these genes, implicating the importance of structural transitions in these DNA tracts in mediating transcriptional regulation of these genes (16–24). In general, DNA unwinding at the proximal promoter region of both prokaryotic and eukaryotic genes is believed to enhance open promoter complex formation with transcriptional machineries leading to transcriptional activation of the genes (26,27). A polypurine/polypyrimidine tract was also found within the proximal promoter region of the VEGF gene, the gene product of which is an angiogenic growth factor. This tract is known to serve as a multiple binding site for Sp1 and Egr-1 transcription factors (13,14). The polypurine tract of the VEGF promoter region contains a guanine-rich sequence consisting of four runs of three or more contiguous guanines separated by one or more bases. This guanine-rich sequence conforms to a general motif capable of forming an intramolecular G-quadruplex:  $G_xN_yG_xN_yG_xN_yG_x$ , where  $x \geq 3$  and  $y \geq 1$  (28,29). Therefore, in this study, we were interested in addressing whether a conformational transition exists among B-type DNA, melted DNA and G-quadruplex structures within this tract in the VEGF promoter, and whether specific G-quadruplex structures can naturally form from the guanine-rich sequence of this tract in the presence of KCl and/or G-quadruplex-interactive agents. We anticipated that the results of this study would enhance our understanding of the role of DNA structure in transcriptional regulation of the VEGF gene. Furthermore, we wished to explore a new therapeutic strategy to treat tumor angiogenesis by repressing the transcriptional activation of a major angiogenic gene, VEGF, by modification or stabilization of non-canonical DNA structures such as G-quadruplexes with G-quadruplex-interactive drugs.

In this report, the dynamic nature of a polypurine/polypyrimidine tract of the VEGF promoter region has been demonstrated through a DNA breathing assay using the



**Figure 8.** CD spectra of the VEGF-Pu20T, d(T<sub>5</sub>G<sub>5</sub>CG<sub>5</sub>C<sub>2</sub>G<sub>5</sub>CG<sub>5</sub>T<sub>5</sub>), in Tris-HCl buffer (20 mM, pH 7.6) in the presence of increasing concentrations of KCl (0, 10, 50 and 100 mM). Each spectrum corresponds to four averaged scans taken at 25°C and is baseline corrected for signal contributions due to the buffer.





Alternatively, the formation of G-quadruplex structures could induce DNA bending at the junction sites between G-quadruplex structures and normal B-DNA structures, potentially leading to the activation or repression of transcription. Future studies are directed toward addressing whether mutation of sequences of the polypurine/polypyrimidine tract could lead to transcriptional repression (or activation) of the VEGF promoter, and if the G-quadruplex-forming ability of this region correlates with the promoter activity of the VEGF genes.

In summary, our results provide strong evidence that specific G-quadruplex structures can naturally be formed by the G-rich sequence within the polypurine/polypyrimidine tract of the VEGF promoter region, raising the possibility that the transcription of this gene can be controlled by ligand-mediated G-quadruplex stabilization.

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*Conflict of interest statement.* None declared.

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