In vitro footprinting of promoter regions within supercoiled plasmid DNA

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Abstract

Polypurine/polypyrimidine (pPU/pPY) tracts, which exist in the promoter regions of many growth-related genes, have been proposed to be very dynamic in their conformation. In this chapter, we describe a detailed protocol for DNase I and S1 nuclease footprinting experiments with supercoiled plasmid DNA containing such the promoter regions to probe whether there are conformational transitions to B-type DNA, melted DNA and G-quadruplex structures within this tract. This is demonstrated with the proximal promoter region of the human vascular endothelial growth factor (VEGF) gene, which also contains multiple binding sites for Sp1 and Egr-1 transcription factors.

Keywords

1. INTRODUCTION

Polypurine/polypyrimidine tracts are known to exist at multiple sites in mammalian genomes, particularly in the proximal promoter regions of growth-related genes (1-7), including VEGF (Fig. 1). These cis-regulatory elements contain multiple Sp1 binding sites and several, studies have independently reported the presence of DNase I- or S1 nuclease-hypersensitive sites within the regions of DNA harboring this tract in both chromatin and negatively supercoiled plasmid DNA, suggesting that this tract is structurally dynamic and easily converted into alternative conformations different from the typical B-DNA structure (8-11). In general, the structural transition of B-DNA to alternative secondary structures is preceded by the local melting or unwinding of duplex DNA, which is facilitated by a negative supercoiling stress naturally generated behind the translocating RNA polymerase complex during the transcription of the genes (12-14). Thus, the structural transition of B-DNA to alternative non-B-conformations is believed to temporarily relieve a negative supercoiling stress generated under normal physiological conditions (15,16). The proximal region of the VEGF promoter contains such a pPu/pPy tract (Fig. 1) and this chapter uses this as an example for examining the structural dynamics, determining whether it can assume a number of different topological forms.

1.1 VEGF

Most primary solid tumors go through a dormant state in which the maximum attainable size is about 1-2 mm in diameter when the tumor cells use only pre-existing host blood vessels (17,18). However, the growth of new blood vessels from pre-existing vessels by a process

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called angiogenesis allows the tumor cells to progressively expand and disseminate to distant organs (17,18). Therefore, angiogenesis represents an essential step for tumor growth and metastasis by providing not only oxygen and nutrients to proliferating tumor cells, but also escape routes for metastatic tumor cells (17,18). The switch to an angiogenic phenotype is mediated by a number of key regulators, such as fibroblast growth factors (FGFs), vascular endothelial growth factors (VEGFs) and angiopoietins, semaphorin, ephrin, Notch/Delta, and the roundabout/slit families of proteins (17,18). Among them, VEGF (or VEGF-A) has been considered to be the key mediator of tumor angiogenesis by stimulating proliferation, migration, survival, and permeability of endothelial cells (19-21).

VEGF expression is mainly regulated at the transcriptional level, and its expression is induced by a variety of factors, including hypoxia, pH, activated oncogenes, inactivated tumor suppressor genes, and growth factors (22-29). VEGF is frequently overexpressed in many types of cancer and the stable expression of VEGF appears to arise from increased VEGF promoter activity (20-30). The VEGF promoter region contains binding sites for several putative 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 (3,22-29). 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 (3).

1.2 Probes for unusual DNA structures

The presence of the pPu/pPy tract within the proximal region of the VEGF promoter (Fig. 1) led us to speculate that this region might be structurally dynamic and could potentially assume a number of different topological forms. Since our previous studies demonstrated that oligonucleotides representing the coding strands of this tract could adopt G-quadruplex structures (30), we further investigated the structural dynamics and the overall forms of the pPu/pPy tract within the promoter of the VEGF gene. We employed *in vitro* footprinting analysis utilizing a negatively supercoiled plasmid DNA containing this region in order to mimic the *in vivo* situation, where negative supercoils prevail due to the active DNA transaction (12-16,30). The footprinting agents used in this study include DNase I, and S1 nuclease, since the reactivity of these probes is very sensitive to the conformation of DNA molecules (31-32). These reagents have been utilized in many previous studies to probe structural transitions from B-DNA to non-B-type DNA structures, such as melted DNA, hairpin structures, G-quadruplex structures, and others (9-11). While DNase I preferentially cleaves locally unwound or normal duplex regions over single-stranded regions, S1 nuclease preferentially cleaves single-stranded regions of DNA over duplex DNA (9-11). However, both enzymes show the lowest cleavage activity toward highly organized secondary structures such as hairpins or G-quadruplex structures (9-11,31,32). For these reasons, the combined use of both nucleases in *in vitro* footprinting experiments reveals pertinent information about unusual structural features of defined elements within the global region of DNA duplex molecules (31,32).

2. MATERIALS

2.1. Labeling 5′-termini of nucleic acids with [^{32}P]

1. T4 polynucleotide kinase (Fermentas).

2. Kinase buffer (10X): 500 mM Tris-HCl (pH 7.6), 100 mM MgCl$_2$, 50 mM DTT, 1 mM spermidine, and 1 mM EDTA.

3. Adenosine 5′-gamma ^{32}P triphosphate (γ-^{32}P ATP), triethylammonium salt (6000 Ci/ mmole, 10 mCi/mL, GE, Healthcare).

4. Micro Bio-Spin™ 30 Columns (Bio-Rad).

2.2. Digestion of plasmid DNA with nucleases

1. DNase I (Promega).

2. S1 nuclease (Promega).

3. A supercoiled plasmid such as pGL3-V789 (*see* Note 1).

4. KCl buffer (10X): 1M KCl, 100 mM Tris-Cl (pH 7.4 at 25 °C) (*see* Note 2).

5. Control buffer (10X): 100 mM Tris-Cl (pH 7.4 at 25 °C).

2.3. Radioactive cycle sequencing and linear amplification

1. Thermo Sequenase DNA Polymerase (USB); 4 units/μl, 0.0006 units/μl *Thermoplasma acidophilum* inorganic pyrophosphatase; 50 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 0.5% Tween®-20, 0.5% Igepal™ CA-630, 50% glycerol.

2. Reaction Buffer (concentrate): 260 mM Tris-HCl, pH 9.5, 65mM $MgCl_2$.

3. Telomestatin (*see* Note 3).

4. Gene-specific primer: 0.5 pmol/μl; (such as VEGF) 5′-CCCAGCGCCACGACCTCCGAGCTACC -3′ (*see* Notes [4-5]).

5. dNTP (10 mM) solution: 10 mM each dATP, dGTP, dCTP, dTTP (*see* Note 6).

6. ddG Termination Mix: 150μM each dATP, dCTP, 7-deaza-dGTP, dTTP; 1.5μM ddGTP.

7. ddA Termination Mix: 150 μM each dATP, dCTP, 7-deaza-dGTP, dTTP; 1.5 μM ddATP.

8. ddT Termination Mix: 150 μM each dATP, dCTP, 7-deaza-dGTP, dTTP; 1.5 μM ddTTP.

9. ddC Termination Mix: 150 μM each dATP, dCTP, 7-deaza-dGTP, dTTP; 1.5 μM ddCTP.

10. Stop Solution: 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol.

2.4. Denaturing PAGE

1. TBE electrophoresis buffer (10X): 0.89 M Tris-HCl (pH 8.0), 0.89 M boric acid, 20 mM EDTA. Store at room temperature.

2. Sixteen percent acrylamide/bisacrylamide (29:1 with 3.3% C) with 8 M urea and N,N,N′,N′- TEMED, Bio-Rad, Hercules, CA.

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[1]Plasmid pGL3-V789 was originally constructed by Dr. Keping Xie by subcloning a 789 bp fragment containing 5′ *VEGF* promoter sequences from −729 to +50 relative to the transcription initiation site into the *Kpn*I and *Nhe*I sites of pGL3-basic (Promega, Madison, WI), which contains firefly luciferase coding sequences (3).
[2]KCl Buffer provides optimum conditions for the formation of G-quadruplex structures from the single stranded DNA.
[3]Telomestatin was kindly provided by Dr. Kazuo Shin-ya.
[4]It is also a good idea to check the sequence of the primer for possible self-annealing (dimer formation could result) and for potential 'hairpin' formation, especially those involving the 3′ end of the primer.
[5]Finally, check for possible sites of false priming in the vector or other known sequence if possible, again stressing matches which include the 3′ end of the primer.
[6]All enclosed reagents should be stored frozen at −20°C and keep all reagents on ice once removed from storage for use.

3. Ammonium persulfate: prepare 10% solution in water. Store at 4 °C up to 1 month.

3. METHODS

In order to test if the pPu/pPy tract of a promoter region is structurally dynamic and can easily adopt a non-B-DNA conformation under physiological conditions, *in vitro* footprinting of the with DNase I and S1 nuclease is performed with a supercoiled form of a plasmid (such as pGL3-V789, which contains the VEGF promoter region from −727 to +50). This plasmid is incubated in the absence of any salt, or in the presence of 100 mM KCl to facilitate the evolution of the secondary structures from the pPu/pPy region. To test the binding of the G-quadruplex interactive agent to the secondary structures, the plasmid DNA is incubated with and without 1 μM telomestatin (33) for 1 hr at 37°C, and then treated with DNase I or S1 nuclease for 2 min. To map S1 and DNase I cleavage sites, linear amplification by PCR was performed with ^{32}P-labeled-gene specific primers to amplify the top strand of both nuclease treated plasmid DNA.

An overall strategy to perform *in vitro* footprinting of the wild-type VEGF promoter contained in a supercoiled plasmid in the presence of K^+ and G-quadruplex-interactive compounds is shown schematically in Fig. 2.

3.1. Isolation of supercoiled plasmids

The supercoiled plasmids are isolated from transformed E. coli strain DH5α using the QIAGEN Plasmid Maxi Kit. This method is based on modified SDS-alkaline lysis of bacterial cells in combination with selective binding of the DNA to silica beads in the presence of certain salts (*see* Note 7).

3.2. Treatment plasmid DNA with nuclease

1. In an empty tube, supercoiled plasmid (2 μg) and 2.5 μl of 10X KCl or control buffer are mixed and brought to 25 μl with the addition of DDW (*see* Note 8).

2. The incubation proceeds at 37°C for over 12 h or overnight to allow the secondary structures to evolve from the pPu/pPy region.

3. For testing the drug binding to the secondary structures, add 1 μl of diluted drug solution in KCl or control buffer (e.g., 25 μM telomestatin in KCl or control buffer) to 25 μL DNA solution and mix them by vortexing and centrifuge briefly.

4. Incubate the reaction mixture at 37°C for 1h.

5. Add 2 μL of diluted DNase I (0.2 U) or 200 U of S1 nuclease to the tube, mix gently by pipetting up and down several times, cap the tubes and centrifuge briefly. After 1 minute digestion, add 100 μL of 0.3 M sodium acetate to the reactions followed by DNA precipitation with 2 volumes 100% ethanol and placement at −20°C overnight.

6. Spin in microfuge 30 min and allow pellet to air dry.

7. Resuspend the dried pellet completely in 25 μL of TE buffer.

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[7]The protocol is suitable for obtaining pure plasmid DNA up to 100μg from 30~100 ml bacterial culture grown in LB medium. The culture volume should be reduced to half or less when bacteria grown in rich medium are used.
[8]It is best to prepare one large reaction mix and then aliquot 25 μl into each sample tube.

3.3. Labeling 5′-termini gene-specific primers with [^{32}P]

1. Prepare a reaction mixture (25 μL), containing oligonucleotide (4 μM), 3 μL γ-^{32}P ATP (6000 Ci/mmole, 10 mCi/mL), T4 polynucleotide kinase (10 U), 2.5 μL 10× kinase buffer, and water.

2. Incubate the reaction mixture at 37 °C for 1 h in water bath for labeling 5′-termini of oligonucleotides with γ-[^{32}P]-ATP.

3. After completion of the reaction, use Micro Bio-Spin™ 30 Columns (Bio-Rad) to remove unincorporated radioactive γ-^{32}P ATP (6000 Ci/mmole, 10 mCi/mL) from labeled DNA. The instructions for use of Bio-Spin™ 30 Columns are based on recommendations from the manufacturer. In brief, the reaction mixture (25 μL) is loaded at the top of the column after centrifuging the column at $1,000 \times g$ for 4 min in a swinging bucket and removing the packing buffer. The column is then centrifuged for 4 min at $1,000 \times g$ to collect the purified 5′-end-labeled oligonucleotide in water (*see* Note 9).

3.4. Radiolabeled Primer Cycle Sequencing

1. Label four tubes representing G, A, T and C.

2. Place 4 μl of the ddGTP termination mix in the tube labeled G. Similarly fill the A, T and C tubes with 4 μl of the ddATP, ddTTP and ddCTP termination mixes respectively.

3. In a separate microcentrifuge tube, combine the following: 1 μL of plasmid DNA (20 pmole), 0.5 μL of concentrated reaction buffer, 1.0 μL of labeled primer, 1.0 μL of distilled water and 0.5 μL of Thermo Sequenase DNA Polymerase (*see* Note 10).

4. Transfer it to the PCR tube (from step 1), mix gently by pipetting up and down several times, cap the tubes and place them in the thermal cycler (*see* Note 11).

5. Carry out PCR using cycling conditions consisting of an initial 10-min denaturation step at 94°C, 1 min at 60°C, and 1 min at 72°C, for a total of 42 cycles (*see* Note 12).

6. Add 4 μl of stop solution to each of the termination reactions, mix thoroughly and centrifuge briefly.

3.5. Linear amplification of the plasmid DNA digested with DNase I or S1 nuclease using ^{32}P-labeled primers

1. Place 4 μl of the plasmid DNA digested with DNase I or S1 nuclease from step 3.2 in each PCR tube.

2. In a separate microcentrifuge tube, combine the following: 0.5 μL of 10 mM dNTP solution, 0.5 μL of concentrated reaction buffer, 0.5 μL of labeled primer, 2.0 μL of distilled water and 0.5 μL of Thermo Sequenase DNA Polymerase (*see* Note 9).

3. Transfer it to the PCR tube (from step 1), mix gently by pipetting up and down several times, cap the tubes and place them in the thermal cycler (*see* Note 13).

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[9]No further purification is required for most sequencing.
[10]Use 1.0 pmol of fresh ^{32}P-labeled primers, but 2-5-fold more primer can be used for shorter exposure times.
[11]It is best to prepare one large reaction mix and then aliquot 4 μl into each sample tube.
[12]The specific cycling parameters used will depend on the primer length and sequence and the amount and purity of the template DNA.
[13]It is best to prepare one large reaction mix and then aliquot 4 μl into each sample tube.

4. Carry out PCR using cycling conditions consisting of an initial 10-min denaturation step at 94°C, 1 min at 60°C, and 1 min at 72°C, for a total of 42 cycles (*see* Note 14).

3.6. Separation of PCR products on denaturing PAGE

1. Set up a denaturing 10% polyacrylamide gel of 30 cm × 30 cm × 0.4 mm.

2. Prepare 60 mL of gel solution by mixing 6 mL TBE buffer (10X), 15 mL of 40% acrylamide/bisacrylamide (29:1), and 30 g urea and adding water to 60 mL. After adding 100 μL ammonium persulfate solution and 20 μL TEMED, pour the gel and insert the comb.

3. Once the gel is polymerized, carefully remove the comb, and wash the well with TBE buffer (1×) using a pasture pipette.

4. Attach the gel plates to the electrophoresis apparatus, and fill both reservoirs of the electrophoresis tank with 1× TBE. Pre-run and warm the gel for a least 30 minutes at 1400 V (constant voltage) using a DC power supply.

5. Heat the samples and sequencing ladders at 95 °C for 3 min, and chill the sample on ice before loading. Run the gel at about 1400 V.

6. After the desired resolution is obtained, detach the gel plates from the electrophoresis apparatus, and carefully separate both plates, leaving the gel attached to one plate.

7. Place a piece of thin chromatography paper (DE81) on top of the gel, and slowly pull back on the paper to transfer gels to the paper.

8. Place a piece of Whatman paper (3MM) underneath, and cover the wet gel with plastic wrap on top.

9. Put the gel sandwich in a dryer between a plastic fiber mat and clear plastic sheet, and dry the gel at 80 °C for at least 1 h with a vacuum.

10. Place the dried gel in an X-ray film cassette. Obtain an autoradiogram by exposing the X-ray film to the dried gel. Alternatively, the image can be obtained by exposing the dried gel to the phosphor screen for an appropriate time and scanning the phosphor screen. Fig. 3 is an example of an autoradiogram of a 10% polyacrylamide sequencing gel, showing the results of S1 and DNase I footprinting experiments carried out with a supercoiled pGL3-V789 plasmid.

Acknowledgments

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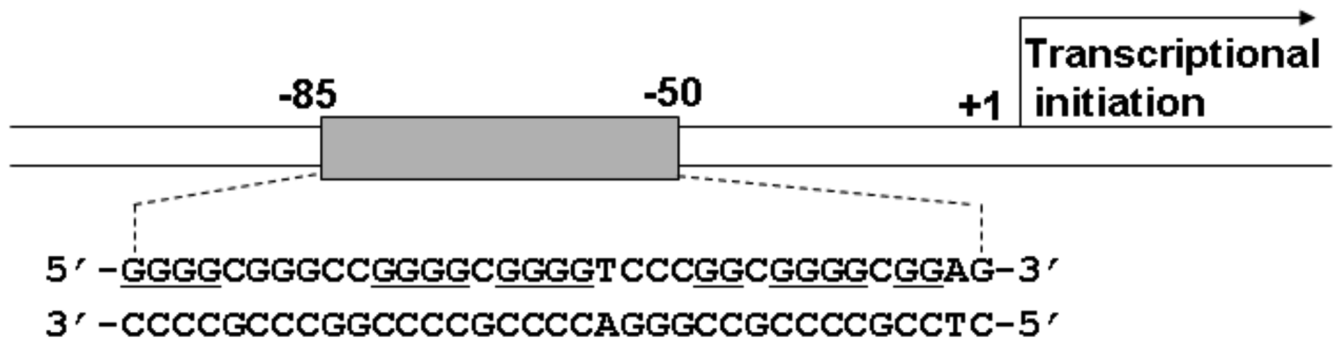
Fig. 1.
Schematic diagram showing the location of the pPu/pPy tract in the proximal promoter region of the VEGF gene.
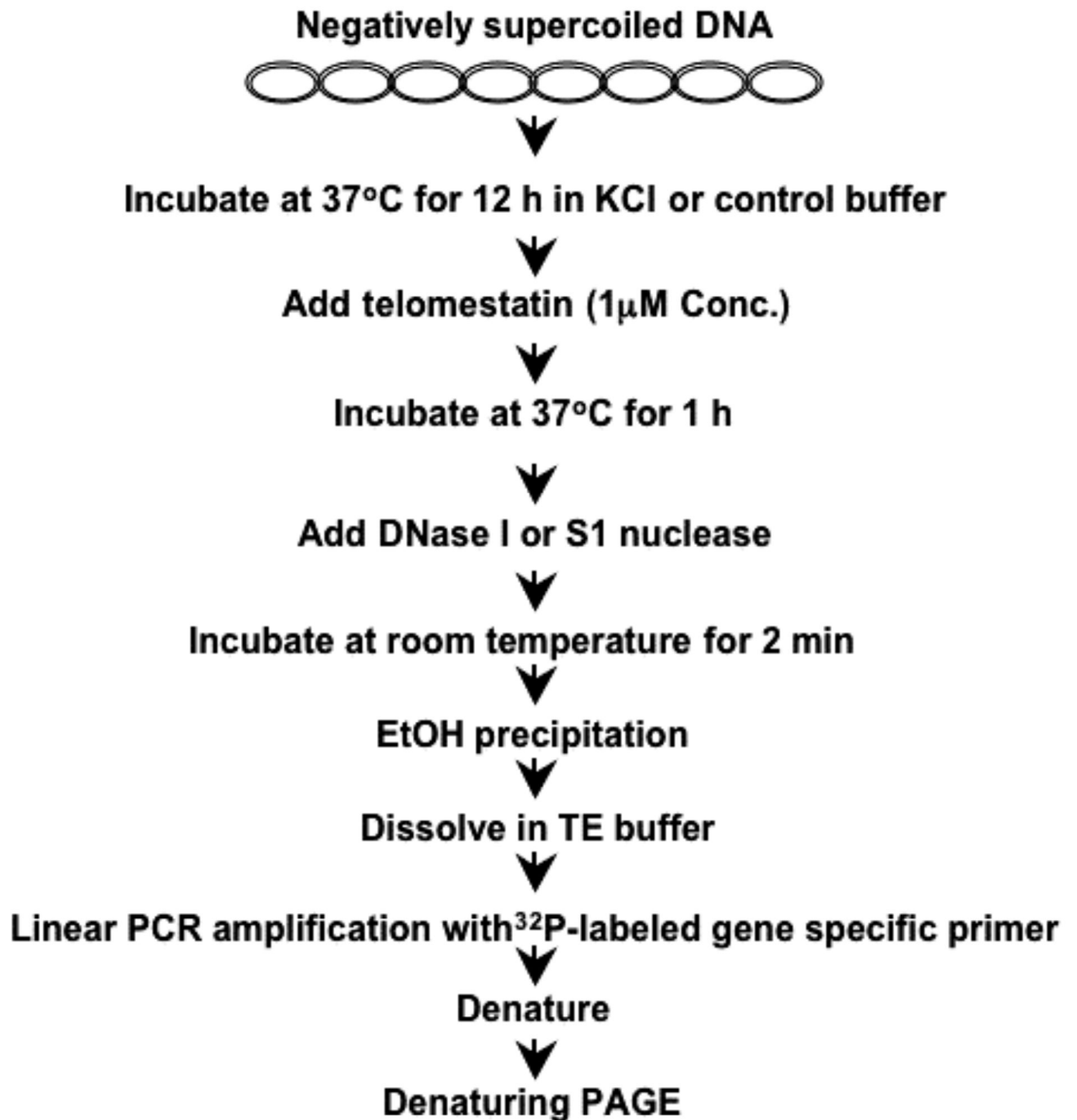
Negatively supercoiled DNA

Incubate at 37°C for 12 h in KCl or control buffer

Add telomestatin (1μM Conc.)

Incubate at 37°C for 1 h

Add DNase I or S1 nuclease

Incubate at room temperature for 2 min

EtOH precipitation

Dissolve in TE buffer

Linear PCR amplification with ^{32}P-labeled gene specific primer

Denature

Denaturing PAGE

Fig. 2.
Flowchart of DNase I and S1 nuclease footprinting experiments of the VEGF promoter region in a supercoiled plasmid.
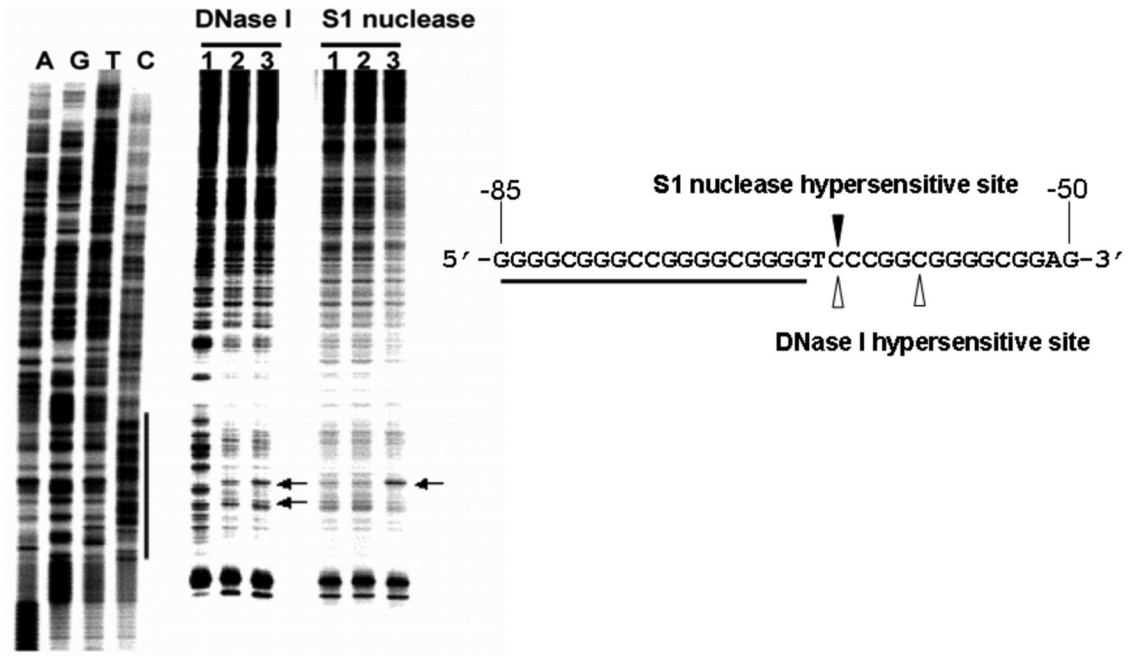
Fig. 3.
In vitro footprinting of the VEGF promoter region with DNase I, S1 nuclease or DMS.
Autoradiograms showing S1 nuclease and DNase I cleavage sites on the G-strand of a
supercoiled pGL3-V789 plasmid. This plasmid was incubated in control (lane 1), or in KCl
buffer without (lane 2) and with 1 μM telomestatin (lane 3) at 37°C for 1 h before digesting
with nucleases. Arrows indicate the hypersensitive cleavage sites to nucleases. The primer
extension reaction revealed a long *protected* region at approximately −53 to −123 bp, including
the G-rich sequences, when a supercoiled pGL3-V789 plasmid was incubated with 100 mM
KCl and digested with DNase I (compare lanes 1 and 2). This indicates a possible transition
from B-DNA to a G-quadruplex structure in the VEGF promoter region, which is consequently
resistant to DNase I digestion. Significantly, a striking hypersensitivity was found in the
presence of KCl and telomestatin at a cytosine located at the 3′-side of the G-quadruplex-
forming region (underlined sequence), which is the junction site separating the putative G-
quadruplex from the adjacent normal B-DNA (see arrow "A" in lane 3). The reactivity of S1
nuclease at the VEGF proximal promoter region was also moderately reduced in the presence
of telomestatin and KCl, and the hypersensitivity site observed with S1 nuclease corresponds
to one of those obtained with DNase I in the presence of telomestatin and KCl (lane 3) (Figure
modified from ref. 30).