

Molecular Structure and Transcriptional Regulation of the Gene for the Platelet-derived Growth Factor α Receptor in Cultured Vascular Smooth Muscle Cells

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Abstract

PDGF has been shown to contribute to hypertrophy in vascular smooth muscle cells (VSMC). PDGF-AA differentially promotes protein synthesis in VSMC from spontaneously hypertensive rats (SHR) but not in those from Wistar-Kyoto rats (WKY). This observation has led us to postulate a role for PDGF α receptor (PDGFR- α) in the hypertensive hypertrophy of blood vessels. Western and Northern blot analyses demonstrated a high and specific expression of the PDGFR- α protein and mRNA in SHR cells but not in WKY cells. To clarify the mechanism of the differential expression of the PDGFR- α gene, we isolated the promoter region of the gene. Studies on the promoter functions indicated that this promoter is active in SHR cells but not in WKY cells. The regulatory domain responsible for this difference was narrowed to the sequence between -246 and -139, which enhanced the promoter activity of SHR fivefold over the basal activity. DNase I footprinting and gel-shift assay indicated that this sequence specifically interact with nuclear proteins from VSMC through the binding site for CCAAT/enhancer-binding proteins, and members of the C/enhancer-binding protein family play a significant role in the strain-specific transcription of the PDGFR- α gene. (*J. Clin. Invest.* 1995, 96:558-567.) Key words: vascular smooth muscle cells • vascular hypertrophy • platelet-derived growth factor α -receptor • promoter activity • CCAAT/enhancer-binding proteins

Introduction

The growth of vascular smooth muscle (vascular remodeling) occurs as an adaptive process in response to chronic changes in hemodynamic conditions and humoral factors. Vascular remodeling has been considered to contribute to pathophysiological changes in cardiovascular diseases. In the context of hypertension, the vascular structural changes in smooth muscle may play an important role in the etiology, amplification, and perpet-

uation of high blood pressure (1). These vascular changes in hypertensive subjects and animals are seen primarily in small arteries and arterioles, although larger vessels also undergo vascular remodeling. Morphologic studies have demonstrated that in the aorta an increase in smooth muscle mass due to hypertrophy rather than hyperplasia is a principal change (2), whereas in smaller resistance vessels hyperplasia may be an important process (3). Extensive studies on the biochemical and cellular mechanisms have been reported. However, factors that initiate and maintain the vascular growth response have not yet been fully clarified. In response to hemodynamic forces, physical injury, or circulating factors, cells in the vessel wall are activated to release growth modulators, cytokines, proteolytic enzymes, and matrix components, thereby participating in the process of vascular remodeling (4).

PDGF is one of the major mitogens in serum and is responsible for proliferation of certain types of cells, including fibroblasts, glial cells, and vascular smooth muscle cells (VSMC)¹ (5, 6). Notably, VSMC are capable of producing and secreting the PDGF-AA homodimer but not its BB isoforms in a growth- and development-dependent manner, which may contribute to the autocrine and/or paracrine growth-stimulating mechanism of blood vessels (7). The action of PDGF-AA is specific to the PDGF α receptor (PDGFR- α) and does not bind the PDGFR- β . In our recent studies, we have reported that the PDGF-BB homodimer is a potent mitogen for cultured VSMC from the normotensive rat strain, Wistar-Kyoto rats (WKY), whereas PDGF-AA is inactive in eliciting DNA or protein synthesis in these cells (8). Given the selective specificity of the PDGF-AA homodimer for the α receptor, we attribute the absence of the response to the AA homodimer to the absence of the α receptor (9). However, we have observed that PDGF-AA promotes protein synthesis in a dose-dependent manner in VSMC derived from spontaneously hypertensive rats (SHR), presumably due to a high level expression of PDGFR- α in the hypertensive rat strain.

Since the action of PDGF-AA (a specific ligand for PDGFR- α) in SHR-derived VSMC was limited to the induction of protein synthesis without an effect on DNA synthesis, we have postulated that PDGFR- α mediates the hypertrophy rather than hyperplasia of VSMC, whereas mitogenic responses of these cells are mediated only through PDGFR- β . Here, we report

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1. Abbreviations used in this paper: C/EBP, CCAAT/enhancer-binding protein; CTF/NF-1, CCAAT-binding transcription factor/nuclear factor I; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; NF-IL6, nuclear factor for IL-6; PDGFR, platelet-derived growth factor receptor; pGLE, pGL2-enhancer vector; pKS(+), pBluescript KS(+) vector; SHR, spontaneously hypertensive rats; SD, Sprague-Dawley; VSMC, vascular smooth muscle cells; WKY, Wistar-Kyoto rats.

evidence for the differential expression of PDGFR- α in VSMC from SHR by examining its binding capacity, the presence of receptor protein, and mRNA. Further, in an approach to clarify the mechanism of the differential PDGFR- α expression, we isolated and characterized the 5'-flanking region containing the promoter and *cis*-acting elements of the PDGFR- α gene. We identified a potential regulatory element that is involved in the differential expression of the PDGFR- α gene in WKY- and SHR-derived VSMC.

Methods

Materials. Recombinant human PDGF isoforms, rabbit anti-mouse PDGFR- α antibodies, and rabbit anti-human PDGFR- β antibodies were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). [125 I]-PDGF-BB (1,000 Ci/mmol) was obtained from Amersham (Arlington Heights, IL), 125 I-goat anti-rabbit IgG and 125 I-goat anti-mouse IgG from ICN (Costa Mesa, CA), and [α - 32 P]dCTP (3,000 Ci/mmol) and [γ - 32 P]ATP (6,000 Ci/mmol) from DuPont/NEN (Boston, MA).

Cell culture. VSMC were isolated from the thoracic aorta of age-matched (12-wk-old) WKY and SHR (Charles River Breeding Laboratories, Wilmington, MA) as described previously (8, 9). VSMC (passages 7–12) were seeded in a dish (10^4 cells/cm 2) and maintained in DME with 10% heat-inactivated FCS at 37°C in a humidified atmosphere of 95% air-5% CO $_2$. In preparation for experiments, confluent cells were made quiescent by placing them in a defined serum-free medium containing insulin (10 μ g/ml), transferrin (10 μ g/ml), and sodium selenite (10 ng/ml) for 2 d. Libby and O'Brien (10) showed that this medium maintains VSMC in a quiescent and noncatabolic state for an extended period of time.

Immunoblotting. VSMC in a 100-mm dish were washed with 20 mM Hepes-NaOH buffer, pH 7.4, containing 130 mM NaCl, 5 mM KCl, 1 mM MgCl $_2$, and 2 mM EGTA and were lysed with 20 mM Hepes-NaOH buffer, pH 7.4, containing 0.5% NP-40, 50 mM β -glycerophosphate, 0.1 mM orthovanadate, 10 μ M molybdate, 1 mM PMSF, 0.1 mg/ml leupeptin, and 10 mg/ml aprotinin at 4°C. After centrifugation at 14,000 g for 5 min, supernatant was used as cell lysate. SDS-PAGE was done using a 7.5% gel according to Laemmli (11), and proteins in the gel were transferred to a nitrocellulose membrane by electroblotting. The membrane was treated with antibodies against PDGFR- α or PDGFR- β , and immunoreactive proteins were detected by autoradiography using goat anti-rabbit or anti-mouse IgG labeled with 125 I.

Competitive binding analysis. VSMC in a 13-mm well (3.5×10^5 cells/well) were washed twice with DME containing 10 mM Hepes-NaOH buffer, pH 7.4, and 0.5% BSA and were incubated with [125 I]-PDGF-BB (1 ng, 69,000 cpm) in the presence of unlabeled PDGF-AA or -BB in 1 ml of the same medium for 3 h at 4°C. After washing five times with the medium at 4°C, cells were lysed directly in 20 mM Hepes-NaOH buffer, pH 7.4, containing 1% Triton X-100 and 10% glycerol, and radioactivity associated with cells was counted.

cDNA probes. PDGFR- α cDNA probes corresponding to the nucleotide positions +796 to +1,432 (probe 1) and -127 to +5 (probe 2) of the rat PDGFR- α cDNA sequence (12) were prepared from the total RNA of rat lung L2 cells (CCL149, American Type Culture Collection) by the reverse transcriptase-PCR method, and the former was used for Northern analysis and the latter for screening a genomic DNA library. A rat PDGFR- β cDNA fragment corresponding to the nucleotide position +1,432 to +2,216 of the mouse cDNA sequence (13) was also prepared from the total RNA of L2 cells by the reverse transcriptase-PCR method. A 1.3-kb fragment of the rat glyceraldehyde 3-phosphate dehydrogenase (G3PDH) cDNA was generated by using the rat G3PDH Control Amplifier Set (Clontech Laboratories, Inc., Palo Alto, CA). The human PDGF A chain cDNA EcoRI fragment (1.3 kb) was kindly made available by Dr. C. Betsholtz. DNA probes were labeled with [α - 32 P]dCTP using the random oligonucleotide method.

Library screening and DNA sequencing. Methods of screening a recombinant genomic library, Southern blot transfer, hybridization to DNA on filters, subcloning into plasmid vectors, and gel electrophoresis were performed according to the procedures described previously (14, 15). A lambda DASH II rat genomic library obtained from the testes of male Sprague-Dawley (SD) rats (Stratagene, La Jolla, CA) was screened by a 133-bp fragment of the rat PDGFR- α cDNA (probe 2) as a probe. One clone that contained a 16-kb genomic sequence of the rat PDGFR- α gene was isolated from $\sim 1 \times 10^6$ clones. A 6.0-kb BamHI fragment of this clone was subcloned into the pBluescript KS (+) vector (pKS(+), Stratagene), which was designated pKS6.0a, and partial sequences of this fragment were determined by the dideoxy chain termination method on a double-stranded DNA template (15).

Northern and primer extension analyses. Total cellular RNA was isolated from VSMC using the LiCl/urea method as described previously (16). For Northern analysis, RNA was electrophoresed in a 1.0% agarose/2.2 M formaldehyde gel and transferred to a nylon membrane (Hybond-N $^+$, Amersham) after staining with ethidium bromide to verify the relative quantity and quality of RNA. The membrane was prehybridized and hybridized by standard techniques (14, 15). After high-stringency washing for 1 h at 60°C, blots were exposed to an x-ray film with an intensifying screen at -80°C. Developed films were scanned by an image scanner (ES-800C Scanner, Epson America, Inc., Torrance, CA) and analyzed by a computer program (NIH Image 1.49) to measure the relative intensity of bands. Primer extension analysis was carried out essentially as described previously (15). For the primer extension analysis of PDGFR- α mRNA, a reverse complement primer (5'-AGCCCCAAGCCTCCAGTCCT-3') corresponding to the nucleotide position -87 to -68 of the PDGFR- α cDNA (12) was synthesized. For the primer extension analysis of promoter-luciferase fusion mRNA, a reverse complement primer (5'-CTTTATGTTTTGGCGTCTTCCA-3') corresponding to the nucleotide position +2 to +24 of the luciferase cDNA was synthesized. These primers were labeled with [γ - 32 P]ATP and hybridized overnight at 42°C with RNA samples. After precipitation with ethanol, pellets were resuspended in a 20- μ l reaction mixture with 50 U of avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI) and incubated for 2 h at 42°C. Products were analyzed by resolution on a 6% polyacrylamide/8 M urea gel. The gel was dried and exposed to an x-ray film with an intensifying screen at -80°C.

Construction of PDGFR- α -luciferase expression vectors. A 1,703-bp genomic sequence of the PDGFR- α gene containing a promoter fragment (1,635 bp) and a partial sequence of the first exon (68 bp) was amplified by PCR using specific oligonucleotide primers (P1, 5'-GACTTGCTTTTAAACAACAGC-3', and P2, 5'-CTCCCTCAAGCT-CCAACAGT-3') (see Fig. 5), subcloned into the EcoRV site of pKS(+), and sequenced. To generate promoter-reporter expression vectors, a SacI-XhoI fragment of the resultant plasmid was subcloned into the promoterless pGL2-enhancer vector (pGLE, Promega) that contained a cDNA of firefly luciferase with a simian virus 40 enhancer sequence. Subsequently, the resultant plasmid, which was designated pLuc-a1, was digested with appropriate restriction enzymes to obtain 5' deletions, pLuc-a2 through pLuc-a7 (see Fig. 6).

DNA transfection and luciferase assay. Promoter-reporter expression vectors were prepared by alkaline lysis of bacterial cultures, purified using a precipitation protocol by the polyethylene glycol method (14), and used for the transfection of VSMC. The smooth muscle cells were seeded in 60-mm dishes (5×10^5 cells/dish) 24 h before transfection. Transfections were performed with cells at $\sim 70\%$ confluency by the DEAE-dextran method (15). After washing twice with PBS, cells were incubated for 15 min at room temperature with DNA mixtures of 3 μ g of the PDGFR- α -luciferase expression vectors and 6 μ g of pSV β -galactosidase control vector (Promega) in the presence of 0.5 mg/ml DEAE-dextran solution in PBS. After the transfection, cells were rinsed gently twice with PBS and incubated for an additional 48 h in the culture medium and then washed twice with PBS, and enzyme assays were performed. Washed cells were incubated for 5 min in a 250- μ l lysis buffer containing 25 mM Tris, pH 7.8, 2 mM EDTA, 2 mM DTT, 10%

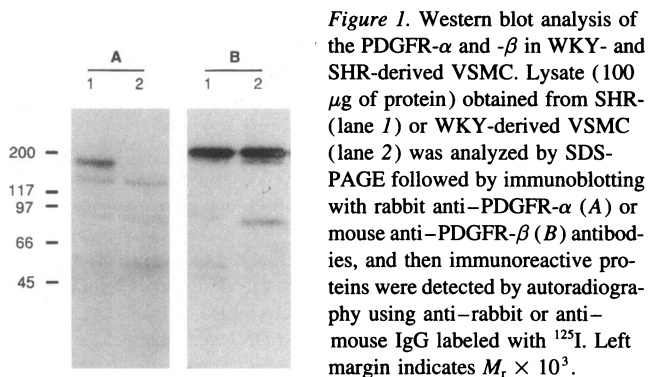


Figure 1. Western blot analysis of the PDGFR- α and - β in WKY- and SHR-derived VSMC. Lysate (100 μ g of protein) obtained from SHR- (lane 1) or WKY-derived VSMC (lane 2) was analyzed by SDS-PAGE followed by immunoblotting with rabbit anti-PDGFR- α (A) or mouse anti-PDGFR- β (B) antibodies, and then immunoreactive proteins were detected by autoradiography using anti-rabbit or anti-mouse IgG labeled with 125 I. Left margin indicates $M_r \times 10^3$.

glycerol, and 1% Triton X-100. Cell lysate was scraped with a rubber policeman, transferred to 1.5-ml microcentrifuge tubes, and spun at 12,000 rpm for 10 min. Supernatant was transferred to new tubes, and directly used for luciferase and β -galactosidase assays. A glass tube containing 20 μ l of supernatant was placed in a luminometer (Optocomp I luminometer, MGM Instruments Inc., Hamden, CT), 100 μ l of 470 μ M luciferin was added automatically, and integrated peak luminescence was measured over a 45-s window after a 5-s delay. The activity of β -galactosidase was determined by absorbency at 405 nm in a spectrophotometer after a 150-min incubation of 100 μ l cell lysate with 100 μ l of 2 \times assay buffer (200 mM Na_2PO_4 , 90 mM β -mercaptoethanol, and 8 mg/ml *O*-nitrophenol- β -D-galactopyranoside) and was used to normalize for variations in transfection efficiency.

Preparation of nuclear extracts. Nuclear extracts were prepared according to the method described by Dignam et al. (17) from quiescent VSMC derived from WKY or SHR. Protein concentrations were determined using the Bio-Rad Protein Assay Reagent (Bio-Rad Laboratories, Richmond, CA), and nuclear extracts were divided into aliquots, quickly frozen in liquid nitrogen, and stored at -80°C .

DNase I footprinting. DNase I footprinting was performed according to the method described by Jones et al. (18) with minor modifications. A 60-bp double-stranded oligonucleotide corresponding to the nucleotide position -197 to -138 of the PDGFR- α promoter (5'-GTTGTTGTT-GAAGTCTGGGGTTGGGATTGGCCCCCAGATTGCATAAAGAGCAAAAAGCCA-3') was subcloned into the EcoRV site of pKS (+) and sequenced. To generate a probe labeled at one end, the resultant plasmid was digested with XhoI or XbaI, end-labeled with [α - 32 P]dCTP using Klenow fragment, and a second digestion was performed with XbaI or XhoI, respectively. Finally, a coding- and noncoding-strand probe (124 bp each) was used for DNase I footprinting and the G + A reaction of the Maxam-Gilbert sequencing technique (15). Nuclear extracts (0, 5, 10, and 20 μ g) were incubated with $\sim 2.0 \times 10^4$ cpm of the probe for 30 min at room temperature in a 40- μ l binding buffer containing 12 mM HEPES-KOH, pH 7.9, 60 mM KCl, 4 mM MgCl_2 , 1 mM EDTA, 1 mM DTT, 10% glycerol, and 50 μ g/ml of poly (dl-dC)·(dl-dC) (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). The reaction mixture was then subjected to treatment with DNase I (0.2 U of RQ1 RNase-free DNase) (Promega) for 1 min at room temperature. Reaction was stopped by adding 100 μ l of 0.3 M sodium acetate, 20 mM EDTA, 0.2% SDS, and 200 μ g/ml of yeast tRNA. The samples were extracted twice with phenol-chloroform, ethanol precipitated, and finally analyzed by resolution on a 7% polyacrylamide/8 M urea gel. The gel was dried and exposed to an x-ray film with an intensifying screen at -80°C .

Gel-shift analysis. Double-stranded oligonucleotides used for the gel-shift analysis were 5'-CCCAGATTGCATAAGAGCAAAAAGCCA-3' corresponding to the nucleotide position -165 to -138 of the PDGFR- α promoter containing an enhancer core sequence for CCAAT/enhancer-binding proteins (C/EBPs) and 5'-CCTTTGGCATGCTGCCAATATG-3' containing a consensus sequence for CCAAT-binding transcription factor/nuclear factor I (CTF/NF-I) (Promega) that was

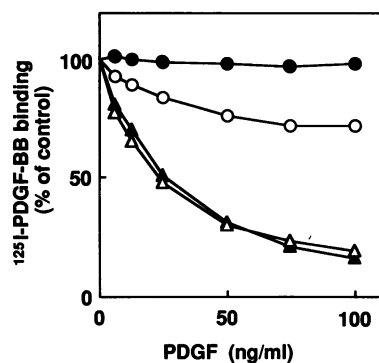


Figure 2. Binding competition by unlabeled PDGF-AA and -BB for [125 I]PDGF-BB in WKY- and SHR-derived VSMC. After WKY- (●, ▲) and SHR-derived (○, △) VSMC were incubated with [125 I]PDGF-BB in the presence of unlabeled PDGF-AA (●, ○) or PDGF-BB (▲, △), cells were lysed directly, and radioactivity associated with cells was counted. Nonspecific binding was determined in the presence of unlabeled PDGF-BB at 500 ng/ml. The binding in the absence of unlabeled ligand (5,600 cpm/well in VSMC from WKY and 9,788 cpm/well in those from SHR) was set at 100%. Data are presented as means of four determinations (SE < 10%).

used as an unrelated competitor. C/EBP oligonucleotide was end-labeled with [γ - 32 P]ATP using T_4 polynucleotide kinase and used as a probe. DNA-protein binding was performed under the same conditions described for DNase I footprinting except that 2 μ g of nuclear extracts was incubated with $\sim 1.0 \times 10^4$ cpm of the labeled C/EBP probe and the reaction volume was 10 μ l. For the competition experiments, a 200-fold molar excess of unlabeled C/EBP or CTF/NF-I oligonucleotide was added to the reaction mixture and incubated on ice for 15 min before adding the labeled C/EBP probe. After incubating with labeled probe for 30 min at room temperature, the samples were analyzed by 4% PAGE under nondenaturing conditions at 4°C in a low-ionic strength buffer containing 6.7 mM Tris-HCl, pH 7.5, 3.3 mM sodium acetate, and 1 mM EDTA. The gel was dried and exposed to an x-ray film with an intensifying screen at -80°C .

Results

Differential expression of PDGFR- α in WKY and SHR cells.

Expression levels of PDGFR- α and - β were compared between WKY- and SHR-derived quiescent VSMC by immunoblotting. Cell lysate obtained from these cells was analyzed by SDS-PAGE followed by immunoblotting with anti-PDGFR- α or - β antibodies (Fig. 1). One major band with a molecular mass of 180 kD and one minor band of 160 kD corresponding to PDGFR- β were observed in cells from both strains of rats (Fig. 1 B), whereas an immunoreactive band with an M_r of 170 kD corresponding to the mature form of PDGFR- α was observed only in VSMC from SHR but not in those from WKY (Fig. 1 A). Competitive binding analysis to ascertain the expression of functional receptors was done to displace the binding of [125 I]PDGF-BB, which binds both to PDGFR- α and PDGFR- β (Fig. 2). Binding of labeled ligand decreased with an increasing concentration of unlabeled PDGF-BB in WKY- and SHR-derived VSMC. The K_d values for PDGF-BB were estimated to be 18 ng/ml both in WKY- and SHR-derived VSMC. The binding sites for PDGF-BB in WKY- and SHR-derived VSMC were estimated to be 100,000 and 170,000 sites/cell, respectively. Unlabeled PDGF-AA failed to displace labeled PDGF-BB in VSMC from WKY, indicating that PDGF-AA does not bind to WKY-derived VSMC. This observation indicated that WKY-derived VSMC did not have PDGFR- α or expressed it at a very low level. In contrast, unlabeled PDGF-AA resulted in a clearly

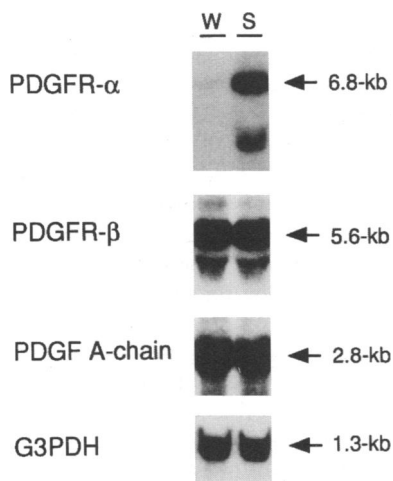


Figure 3. Northern blot analysis of PDGFR- α , - β , and PDGF-A mRNAs in WKY- and SHR-derived VSMC. Total RNA (20 μ g) obtained from quiescent WKY- (W) or SHR-derived VSMC (S) was analyzed by Northern blotting for PDGFR- α , - β , and PDGF-A mRNAs. Expression levels of G3PDH mRNA were used as internal control to standardize the amount of total RNA actually blotted onto a membrane.

recognizable decrease in the binding of [125 I]PDGF-BB in a concentration-dependent manner in VSMC from SHR, and the binding sites for PDGF-AA were estimated to be 51,000 sites/cell. Steady state mRNA levels of PDGFR- α and - β were also compared between VSMC from WKY and SHR by Northern analysis (Fig. 3). Although both WKY- and SHR-derived VSMC expressed PDGFR- β mRNA (5.6 kb), PDGFR- α mRNA (6.8 kb) was expressed only in VSMC from SHR at a high level. PDGFR- α gene transcription was absent in WKY-derived VSMC. In addition, VSMC derived from another normotensive rat strain, SD rats, also did not express PDGFR- α mRNA, whereas rat pheochromocytoma cell line, PC12W cells, expressed PDGFR- α mRNA \sim 10-fold higher than SHR-derived VSMC (data not shown). The differential expression of PDGFR- α was confirmed with three pairs of VSMC preparations. PDGF-A mRNA (2.8 kb) was expressed in both WKY- and SHR-derived VSMC at comparable levels.

5'-Flanking region of the rat PDGFR- α gene. Since there are significant differences in PDGFR- α mRNA levels, which may reflect differences in its transcription between WKY- and SHR-derived VSMC, the 5'-flanking region was isolated and analyzed to study the strain-specific transcriptional regulation of the PDGFR- α gene. One genomic clone of the PDGFR- α gene (\sim 16 kb) was isolated from an SD rat genomic library and characterized by Southern blotting and partial DNA sequence analysis. Since a 6.0-kb BamHI fragment of this clone contained the 5'-flanking sequence, the entire first exon, and a part of the first intron, this fragment was subcloned into pKS(+). The resultant plasmid (pKS6.0a) was used for subsequent studies on the structure and function of the PDGFR- α promoter. By primer extension analysis using the reverse complement primer of the sequence corresponding to the nucleotide position -87 to -68 of PDGFR- α cDNA (12), two major transcriptional initiation sites were identified in VSMC from SHR (Fig. 4, lane 2, #1, adenine residue, and #2, guanine residue). Fig. 5 shows a sequence alignment of the PDGFR- α gene that contains a 1,645-bp stretch of the 5'-flanking region and 169 bp of the first exon and is numbered starting from the proximal transcription initiation site (adenine residue, in Fig. 4 as +1). To compare the promoter structure between WKY and SHR genomes, the segment flanked by the primers, P1 and P2, were amplified

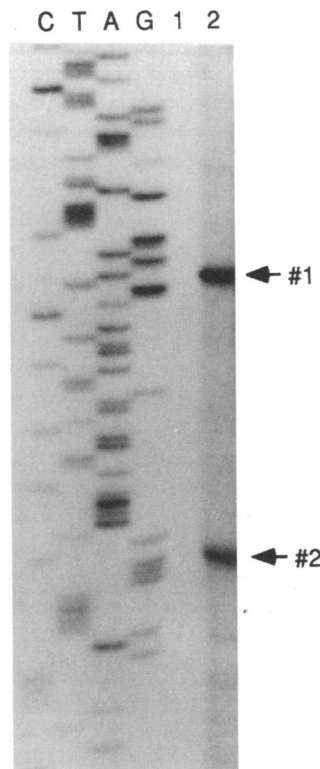


Figure 4. Determination of the transcription initiation sites by the primer extension analysis. Total RNA from SHR-derived VSMC was hybridized overnight at 42°C with a [γ - 32 P]-labeled 20-mer oligonucleotide primer and extended with reverse transcriptase. Reaction products were resolved on a 6% polyacrylamide sequencing gel before autoradiography. Lanes C, T, A, and G are sequence ladders from pKS6.0a double-stranded template using the same primer; lane 1, primer extension from 20 μ g yeast tRNA, and lane 2, primer extension from 20 μ g total RNA of SHR-derived VSMC. #1, adenine residue, and #2, guanine residue, indicate two major transcription initiation sites of the PDGFR- α gene.

from genomic DNAs of the two strains by PCR and subcloned into the EcoRV site of pKS(+). Sequence analyses of these fragments revealed that promoter sequences obtained from WKY and SHR genomic DNAs were identical to the sequence of SD rats as shown in Fig. 5.

A computer-assisted search was performed for exact matches with well-defined transcriptional regulatory elements. Although sites for interaction with the RNA polymerase II transcription complex such as a TATA box or GC box (Sp1) were not found in the 5'-flanking region of the PDGFR- α gene, several potential transcriptional elements were seen to cluster around the sequence within 500 bp from the proximal transcription initiation site. Notable sequences in this region that perfectly match the consensus elements include a protein kinase C-responsive element (AP-1) found at position -128 bp; five AP-2 enhancer elements at positions -206, -226, -330, -452, and -472 bp; an enhancer core sequence for C/EBP at position -159 bp; a cAMP responsive element at position -251 bp; and three binding sites for nuclear factor for IL-6 (NF-IL6) at positions -159, -300, and -397 bp. Other regulatory elements found in a distal sequence from -500 to -1,600 bp include a cardiac muscle-specific enhancer found at position -550 bp; a metallothionein responsive element at -621 bp; two GATA-1 boxes (GATA) at -698 and -726 bp; two binding sites for NF-IL6 at -831 and -1,199 bp; three AP-2 elements at -1,068, -1,221, and -1,282 bp; and a binding site for nuclear factor for κ B at -1,328 bp. In addition, an alternating purine-pyrimidine sequence of the d(TG) $_n$ type with 30 repeats is found at position -1,592 bp.

Functional analysis of the 5'-flanking region. Seven luciferase expression vectors containing 5' deletions of various lengths over a 1.6-kb stretch of the PDGFR- α promoter region were

-1,645 CCTAA ATCTTGACTT GCTTTTAAACA
 -1,620 ACAGCTGAAA GCCCTCCACC CCTGCTCCCT GTGAGAGAGT GTGAGAGAGT GTGAGAGAGT
 -1,580 GTGAGAGAGT GTGAGAGAGT GTGAGAGAGT ACGCGCTCCG AGGAGTCGTT AGCATCCTGT
 -1,500 TCATTATCTA CAAACATTAC GGTATCTCGC TAGCATTCCG AACAAAGGGC TGTTCATACA
 -1,440 TTTCCGCTTC AATGATTTC TGAAGAACA TGTGGAAGTA ATTGGGAGCA GGGCAGTCAC
 -1,380 CCGAGCTGAA GATACACCGA GAGCGGGCG TGCAGAAGG CATAGGAGAA TTGGACTCT
 -1,320 CGGCCAGCT CCAGACTCC TCTTCCGAA CCTGACACC CAAGGTCGG GTCCTATTTA
 -1,280 GGCACAGCAC CCGACTTTC GGGAAACCTC TGTGATTAC CCAGGCTCA GGGCAGCTGC
 -1,200 GTGGTGAAT AACCACTGGT TATTCGAGT TTTGTTATCT ACCTGACCCG CTTGTCCATT
 -1,140 TTAATTCGGA TATGAGTAG ATGGAGTAG TGTGTGTTG GGGGTGGTG TGAGGATAAA
 -1,080 GGACTACTCT CGTCCCGCC CCCCCAAGA GCATTCAAAA AATAGAGCCG AAGTCGAAGG
 -1,020 GCCCTCCCTT CCCCCTACTG CTCTGGGAGG TGCCGTGCGC CCAGGTTTGG TTCCTGGAGT
 -960 TCAGCGCCCG CTTTCCCTCC CGGCAGGATC GCGCGACTC TTCGCGCCTG GGTGTCTGG
 -900 TTTCTGGCT CAAACATCCG GAAAGCTTTC CTAGGTTACC TCGTGTGGGA CCCACTCGGA
 -840 AGGGTGAAT TTAGGAAGT CAAAATACCT CTGCCATCCA GATGCAGAGC GCAGGCGTCC
 -780 CGTAGTACA CCTAGCCTGG GCCTTTTCAT ATGAAGATTG AAGAACAGG GACCAGATAA
 -720 CTCAGAAAAC AAAGGAGTCA CCAGATAAGT GGTCCAAAG GGATAAGGTC GTTCTTCCGG
 -660 AGAACATCCA TCCCAAGGTC ACGCTGAGCA ACCGTTATTT GCACACCATC TCACAATCCA
 -600 GCGCTTCAA AACCCATCAT TTTCTTATTA GGCTCCACAG TTTCTTAATC CCATTAAGG
 -540 ATTACCAACT ACGCGGACTT TCCCTTACG ACCCCAGTT CAAAAGCAGC CAGCCGCATT
 -480 ACGATTCTCT CCCAGGGCA TATTCTTAGC GAGGCCGGGA CTGCCTATAG AAGGAGAAAT
 -420 TTGAATCTA GATTATATAT CGTTTATAG ATAAGCAGTA ATTTTACGTC TGTTTTCCTT
 -360 TCGAAGGGAG TACAAAAACT ATAGCGACAC CCCCCTCCG CCATTGAGAA GTCAACTCAT
 -300 TTTGGAAAT ATGGCTGTTT GAGTTTCATG AACCCCTTCT CCCGGCAGAA CGTCAACACC
 -240 TCCCTTCTCA GCCACCCCA CCCCATCTGG TTTGCCCCC CCCGTTGTTG TTGAAGTCTG
 -180 GGGTTGGGAT TGCCCCCCA GATTCGATTA GAGCAAAAAG CCATGGAGAG GTCTGAGGCT
 -120 GAGAGAGTT CAGAGCAAA GGAGGACTCC GAGGAAACT TTTATTTTGA AGAGACCAGG
 -60 GGGGGGGGAC TTTCTTCTCT GACAGCTAT TACTTTGAGC AAATGATTAG TTTTCCGGAG
 +1 ATGACTATAA CATTGATCA ATTCAAAAAT CGCGGTTTG AGCCCAATTC TGTTGAGCT
 +61 TGAGGGAGT AAACAACAG AGGAACCTGT GCGGAGACT GAGGCTTGG GGTCTACTT
 +121 TCTCCCGGG CTATCGGAT TCTTAGCAA ACTGACCTAG GAGGAGAAG TAAGGGAGAG

Figure 5. The sequence of the promoter region and upstream regulatory elements for the PDGFR- α gene. The first base of the first exon (#1) is indicated as position +1 and the first base upstream from the transcription initiation site is shown as position -1. Position of #2 indicates another transcription initiation site as shown in Fig. 4. The first exon is shown in bold letters. Underlined sequences show the notable regulatory *cis*-acting elements. AP-1 indicates protein kinase C-responsive element; AP-2, AP-2 enhancer element; C/EBP, enhancer core sequence for CCAAT/enhancer-binding proteins; CRE, cAMP responsive element; NF-IL6, binding site for nuclear factor for IL-6; CARg, cardiac muscle-specific enhancer element; MRE, metallothionein responsive element; GATA, GATA-1 box; and NF- κ B, binding site for nuclear factor for κ B. A double-underlined sequence shows alternating purine-pyrimidine dinucleotide repeats of the d(TG)_n-type. Dotted-underlined sequences (P1 and P2) indicate PCR primers used for creating the promoter-reporter expression vector, pLuc-a1 (Fig. 6), and for amplifying the promoter regions from WKY and SHR genomic DNAs. The nucleotide sequence has been submitted to GenBank/EMBL Data Bank, accession number U13172.

prepared by the ligation of restriction enzyme fragments into the luciferase cDNA reporter plasmid, pGLE. The deletion mutants had nucleotide sequences starting at positions -1,635, -1,381, -877, -696, -358, -246, and -139 bp upstream from the transcription initiation site (Fig. 6). To confirm that the transcriptional activity of the PDGFR- α promoter-luciferase expression vectors was mediated by PDGFR- α sequences rather than vector sequences, the transcriptional initiation site of the promoter-luciferase fusion gene was mapped by primer extension analysis (Fig. 7). Using SHR-derived VSMC transfected

with pLuc-a2, the total RNA extracted from the transiently expressed cells produced a prominent band at the adenine residue corresponding to the proximal transcription initiation site for the PDGFR- α gene (Fig. 4, lane 2, #1).

The functional promoter activity of these 5' deletions was assessed by their ability to drive luciferase cDNA expression using VSMC from WKY and SHR. After normalization for transfection efficiency in reference to spectrophotometrically determined β -galactosidase activity, the luciferase activity of pGLE alone was subtracted from those of expression constructs containing 5' deletions. The correction for the baseline level activity was done individually for WKY- and SHR-derived VSMC. Promoter activities thus corrected are presented in Fig. 8 as relative luciferase activity in reference to the maximum activity observed in VSMC from WKY that was set as unity. When each of the 5' deletion constructs was transfected into VSMC from WKY, it drove luciferase transcription at a very low level compared with those from SHR. The promoter activities of the 5' deletions in SHR-derived VSMC were 3- to 18-fold higher than those observed in WKY-derived VSMC. In addition, this promoter was also inactive in VSMC derived from another normotensive rat strain, SD rats, that did not express the PDGFR- α , whereas it showed a significant activity (two- to threefold higher than that observed in VSMC from SHR) in PC12W cells that expressed PDGFR- α at a very high level (data not shown). In VSMC from SHR, a 139-bp subfragment upstream from the first exon (pLuc-a7) consistently drove the luciferase transcription to a level threefold higher over that seen from the promoterless construct, pGLE. Particularly, the sequence spanning -246 through -139 enhanced the expression of luciferase about fivefold over the basal activity observed in SHR-derived VSMC (pLuc-a6 vs. pLuc-a7). On the other hand, the sequence spanning -1,635 through -1,381 reduced the luciferase activity by 60% (pLuc-a1 vs. pLuc-a2).

Differentially regulated cis-acting element of the PDGFR- α promoter. To locate potential *cis*-acting elements that are critically related to the difference in transcriptional activity between WKY- and SHR-derived VSMC, DNase I footprinting was performed on the region between pLuc-a6 and pLuc-a7 (Fig. 9). A coding- or noncoding-strand probe containing the sequence spanning -197 through -138 was end labeled and incubated with nuclear extracts prepared from quiescent VSMC derived from WKY or SHR. Regions protected from DNase I were evident in both coding- and noncoding-strand experiments, reflecting the interaction of *trans*-acting factors at this site. Nuclear extracts obtained from WKY- and SHR-derived VSMC recognized an identical region spanning -163 through -139 (5'-CCAGATTGCATAAGAGCAAAAAGCC-3') on a coding-strand or -164 through -151 (5'-GGGTCTAACGTATT-3') on a noncoding-strand, respectively. The footprinted region on each strand contained an enhancer core sequence for C/EBP as shown in bold letters. Additionally, the level of binding activity in nuclear extracts obtained from SHR-derived VSMC was approximately fourfold higher than that from WKY-derived VSMC.

To further address the nature of the binding activity seen in the footprinted regions, a gel-shift assay was performed by using synthetic double-stranded oligonucleotides containing an enhancer core sequence for C/EBP (Fig. 10). Nuclear extracts from WKY- or SHR-derived VSMC were reacted with a 28-bp radiolabeled C/EBP probe spanning -165 through -138.

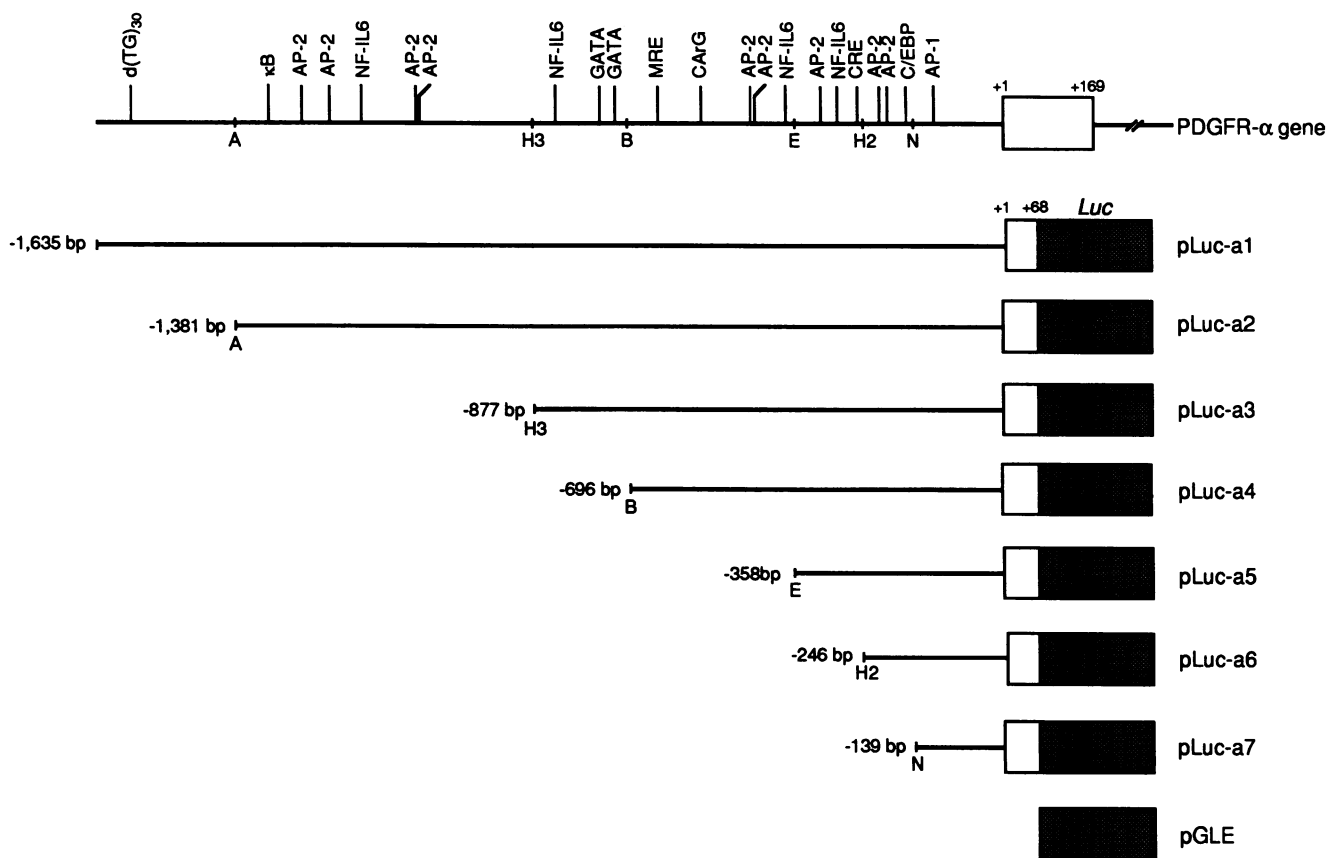


Figure 6. Construction of the PDGFR- α promoter-luciferase expression vectors. A 1,703-bp fragment containing 1,635 bp of the 5'-flanking sequence and 68 bp of the first exon was amplified by PCR using P1 and P2 primers in Fig. 5 and subcloned into pKS(+). A SacI-XhoI fragment of the resultant plasmid was subcloned into pGLE to produce pLuc-a1. As shown schematically, serial 5' deletions of the pLuc-a1 (pLuc-a2 to pLuc-a7) were generated by restriction enzyme digestions as follows. A, Aval; H3, HindIII; B, BamHI; E, EcoRI; H2, HincII; N, NcoI; Luc, luciferase cDNA.

The C/EBP probe was shifted by nuclear extracts from WKY- and SHR-derived VSMC generating specific bands (Fig. 10, lanes 1 and 4). All bands were completely competed out in the presence of a 200-fold molar excess of unlabeled C/EBP oligonucleotide (lanes 2 and 5) but not in the presence of unlabeled CTF/NF-I oligonucleotide (lanes 3 and 6). Nuclear extracts from WKY-derived VSMC generated a single band (lane 1, B3), whereas those from SHR-derived VSMC generated two closely shifted bands (B1 and B2) in addition to B3 (lane 4).

Discussion

Vascular remodeling is considered to be a major mechanism involved in hypertension (19–21). The vasculature of SHR is known to undergo a remodeling presumably by a hypertrophic change in the vascular smooth muscle. In search of molecular and cellular mechanisms for such a change, we found a marked difference in the expression level of PDGFR- α between the VSMC of SHR and normotensive inbred WKY as well as outbred SD rats. In cells derived from the normotensive rats, the PDGFR- α was at a level undetectable by Northern blot, whereas VSMC from SHR expressed it at a level comparable with PDGFR- β , which was expressed both in normotensive and hypertensive rats. The differential α receptor expression is of

pathophysiological significance because its ligand PDGF-AA is also produced in VSMC and can activate an autocrine vascular hypertrophy mechanism that may result in vascular remodeling implicated in the development and maintenance of hypertension (PDGFR- β does not react with PDGF-AA).

To identify molecular bases for the differential expression of PDGFR- α between VSMC from SHR and normotensive rats strains, we determined the nucleotide sequence of the 5'-flanking region of the α receptor gene and found that the transcriptional activity of the PDGFR- α promoter in SHR cells is severalfold higher than that in WKY cells. By DNase I footprinting and gel-shift assay, we have narrowed the *cis*-acting segment responsible for the differential activity to a C/EBP binding site. A gel-shift assay revealed that difference in members of the C/EBP family in the nuclear extracts of SHR and WKY cells may be involved in a mechanism that will account for the differential expression of PDGFR- α between the hypertensive (SHR) and normotensive (WKY and SD) rats strains.

PDGF isoforms are well-known mitogens. Therefore, the autocrine secretion of PDGF-AA in VSMC was expected to stimulate the hyperplastic growth of VSMC. Both PDGFR- α (which responds to PDGF-AA and -BB) and PDGFR- β (specific receptor for PDGF-BB) are capable of mediating a mitogenic response in certain cell types (22–25). However, Heldin

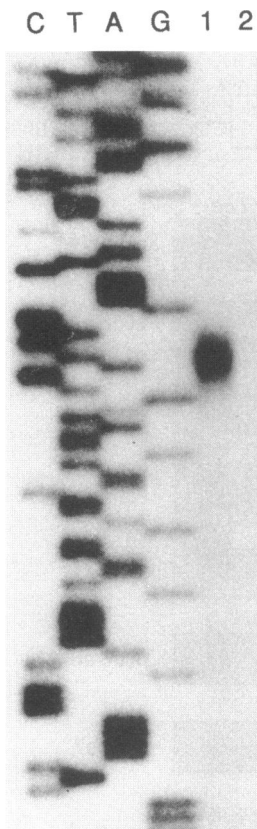


Figure 7. Primer extension analysis of the promoter-luciferase fusion gene. The transcriptional initiation site of the promoter-luciferase fusion gene was mapped by primer extension analysis using a luciferase specific primer under the same conditions as shown in Fig. 4. Lanes C, T, A, and G are sequence ladders obtained from pLuc-a2 double-stranded template using the same primer; lane 1, primer extension with 20 μ g total RNA of the transfected SHR-derived VSMC with pLuc-a2, and lane 2, primer extension with 20 μ g total RNA of the untransfected SHR-derived VSMC. An arrow indicates the transcription initiation site of the promoter-luciferase fusion gene. This initiation site corresponds to the proximal transcription initiation site for the wild-type of the PDGFR- α gene (Fig. 4, lane 2, #1).

et al. (26) and Escobedo et al. (27) have shown that in fibroblasts, which express both PDGFR- α and - β , the PDGF-AA homodimer is considerably less potent a mitogen as compared with the PDGF-BB homodimer or PDGF-AB heterodimer. We have also observed that the action of PDGF-AA is limited to the stimulation of protein synthesis in cultured VSMC without an effect on DNA synthesis and that this effect is seen only in cultured VSMC from SHR but not in those from WKY (9). In our preliminary report (9), we proposed that the strain-dependent vascular hypertrophic action (without hyperplastic action) of PDGF-AA is presumably due to an increase in PDGFR- α expression.

Competitive binding analysis (Fig. 2) showed that SHR-derived VSMC expressed both PDGFR- α and - β at high levels (51,000 and 170,000 sites/cell, respectively), whereas PDGFR- α was absent in VSMC from WKY though PDGFR- β was expressed (100,000 sites/cell). These observations are supported further by a higher level of PDGFR- α mRNA in SHR-derived VSMC compared with that in WKY-derived VSMC in which it is almost absent (Fig. 3). These results strongly suggest that there may be a difference in the transcriptional regulation of the PDGFR- α gene between WKY- and SHR-derived VSMC.

To determine the molecular basis for the difference in the transcription of the PDGFR- α gene between WKY- and SHR-derived VSMC, we isolated and characterized a genomic clone containing the 5'-flanking region of the rat PDGFR- α gene. Recently, the 5'-flanking region of the PDGFR- α gene was isolated from a mouse BALB/c-3T3 fibroblast genomic library (28). Although the nucleotide sequence of its 5'-flanking region has not been published, major organizational features of the rat

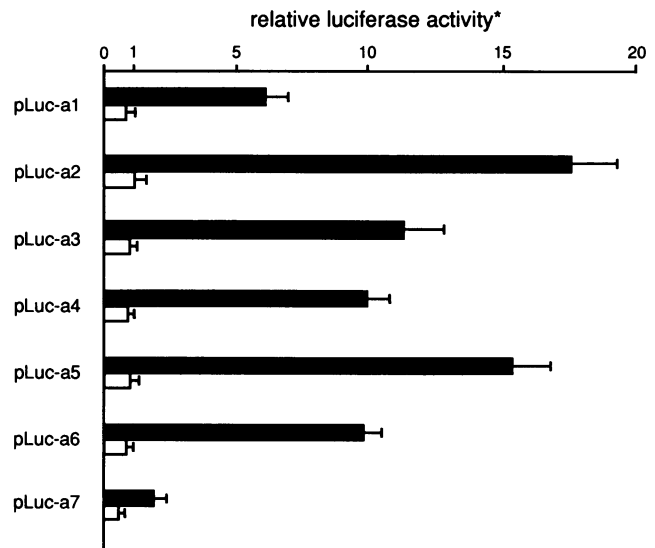


Figure 8. Transcriptional activity in VSMC from the 5' deletions of the PDGFR- α promoter region. The 5' deletions of the PDGFR- α promoter were cotransfected with pSV β -galactosidase control vector to WKY- (\square) and SHR-derived (\blacksquare) VSMC using the DEAE-dextran method. Cell lysate was prepared 48 h after transfection and assayed for luciferase and β -galactosidase activities. After normalization for transfection efficiency in reference to spectrophotometrically determined β -galactosidase activity, the luciferase activity of pGLE alone was subtracted from those of expression constructs containing 5' deletions. Corrections were made individually for WKY- and SHR-derived VSMC. Promoter activities thus corrected are presented as relative luciferase activity in reference to the maximum activity observed in WKY-derived VSMC that was set as unity. The data are expressed as means+SE of four separate assays.

PDGFR- α gene presented herein are in good accord with those of the mouse PDGFR- α gene. Common features between mouse and rat PDGFR- α genes are as follows: the first exon is very short (169 bp in length for the rat), this exon encodes the untranslated region of the message, and the translation initiation codon is located in the second exon that is separated from the first exon by a long intron.

We have determined the nucleotide sequence of the promoter within 1.6 kb of the rat PDGFR- α gene (Fig. 5) and have shown that this region contains several transcriptional regulatory elements, suggesting that it will be a functional promoter of the PDGFR- α gene. The promoter-reporter assay demonstrated that the promoter of the PDGFR- α gene was active only in VSMC from SHR but not in those from WKY (Fig. 8). This promoter was also inactive in VSMC derived from another normotensive rat strain, SD rats, that did not express the PDGFR- α . The promoter structures (within 1.6 kb) obtained from WKY and SHR genomic DNAs were identical to that obtained from SD rats, including the length of the dinucleotide repeats d(TG)₃₀. These observations indicate that the promoter of the PDGFR- α gene is regulated in a strain-specific manner in VSMC and suggest that the strain-specific transcriptional control may be due to differences in *trans*-acting nuclear factor(s) rather than in the structure of the PDGFR- α promoter sequence. Moreover, this promoter showed a significant activity (two- to threefold higher than that observed in VSMC from

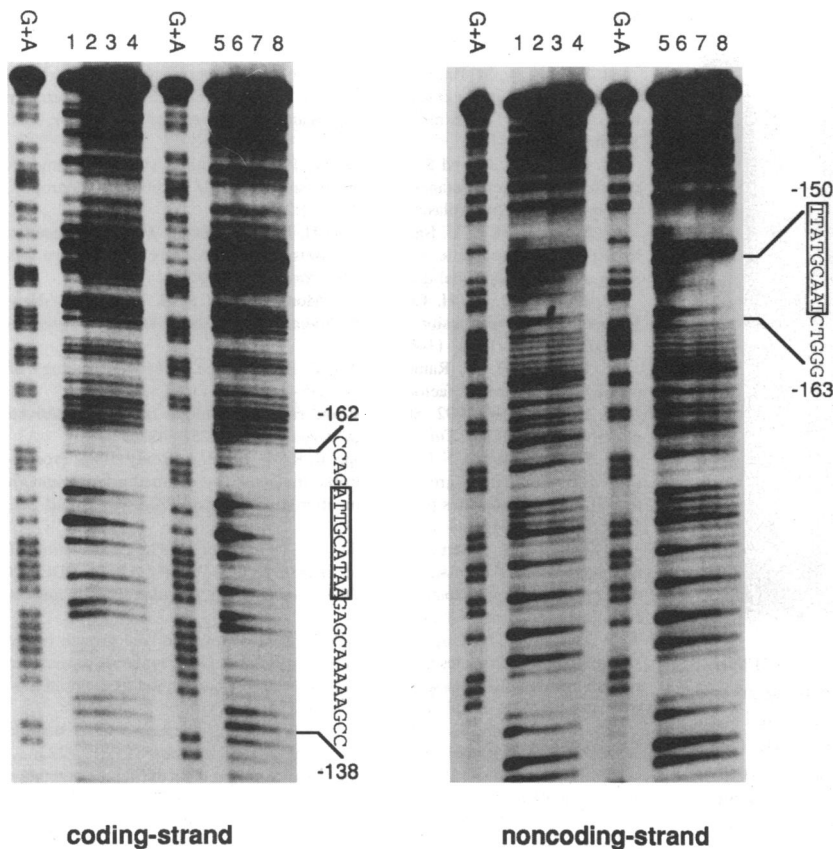


Figure 9. DNase I footprinting of the PDGFR- α promoter using nuclear extracts from VSMC derived from WKY and SHR. The probes cover sequences from -197 to -138 of the PDGFR- α gene promoter and are end labeled on the coding strand at the polylinker site of XhoI or the noncoding strand at the polylinker site of XbaI. Nuclear extracts were incubated with the probe for 30 min at room temperature and then treated with 0.2 U of DNase I for 1 min at room temperature. Reaction products were resolved on a 7% polyacrylamide sequencing gel before autoradiography. Lanes 1 and 5, 0 μ g; lanes 2 and 6, 5 μ g; lanes 3 and 7, 10 μ g; lanes 4 and 8, 20 μ g of nuclear extracts obtained from quiescent VSMC derived from WKY (lanes 1–4) and SHR (lanes 5–8). Lane G + A corresponds to the G + A reaction of the Maxam-Gilbert sequencing technique (15). Sequences to the right represent the observed footprints with numbers indicating their limits and boxes indicate the enhancer core sequence for C/EBP.

SHR) in the rat pheochromocytoma cell line, PC12W, which expressed PDGFR- α at a very high level. This observation suggests that the transcription of the PDGFR- α gene may be regulated by a mechanism related to a tissue or cell-type specificity.

The subfragment from $-1,635$ to $-1,381$ bp showed a 60% inhibition of the highest luciferase activity observed in pLuc-a2. This region contains alternating purine-pyrimidine dinucleotide repeats of the d(TG) $_n$ -type (29–31). The dinucleotide repeats d(TG) $_n$ or d(CA) $_n$ (32) have been shown to exert a negative effect on the transcription of the gene and also downregulate the expression of promoter-reporter fusion gene. Therefore, the distal sequence d(TG) $_{30}$ may play a role in the inhibitory control of PDGFR- α gene expression in VSMC. However, because no polymorphic difference was found between SHR and WKY, it should not be responsible for the strain-dependent differential expression of PDGFR- α . On the other hand, the subfragment from -246 to -139 bp enhanced (fivefold) the promoter activity only in SHR-derived VSMC (Fig. 8, pLuc-6a vs. pLuc-7a). It suggests that this segment may be pivotal in the difference of transcriptional regulation of the PDGFR- α promoter between WKY- and SHR-derived VSMC. DNase I footprinting clearly demonstrated that the sequence spanning -164 through -138 of this region, which contained an enhancer core sequence for C/EBP overlapped with a binding site for NF-IL6, was protected by nuclear extracts obtained from both WKY- and SHR-derived VSMC (Fig. 9). The binding activity of the nuclear extracts from SHR-derived VSMC was fourfold higher than that from WKY-derived VSMC. These results indicate that the binding site for C/EBP is actually used as a potential *cis*-acting

regulatory element in VSMC, and the total amounts of C/EBP in the nuclear extracts from SHR-derived VSMC are fourfold greater than those from WKY-derived VSMC.

C/EBP belongs to a family of the basic region-leucine zipper class regulatory proteins that enable homo- and heterodimerization of C/EBPs (33). In mammalian species, C/EBP family consists of at least five unique members, C/EBP α , C/EBP β (also known as LAP, LIP, CRP2, IL-6DBP, and NF-IL6), C/EBP δ (NF-IL6 β), C/EBP γ (Ig/EBP-1), and CHOP-10 (GADD153) (34–43). Since all known members of C/EBP family bind to similar consensus sequences T(T/G)NNG(C/T)AA(T/G) (44) *in vitro*, gel-shift assay was performed to characterize the nature of the binding activity seen in the footprinted region (Fig. 10). Nuclear extracts from WKY-derived VSMC generated a single binding complex (B3), and the relative intensity of the B3 was almost equal to that observed in nuclear extracts from SHR-derived VSMC. In contrast, two additional binding complexes (B1 and B2) were observed in nuclear extracts from SHR-derived VSMC but not in those from WKY-derived VSMC. These results suggest that C/EBP members generating the B1 and B2 complexes may play an important role in the differential transcription of the PDGFR- α gene in WKY- and SHR-derived VSMC. Originally, C/EBP was isolated from rat liver nuclei as a sequence-specific DNA-binding protein with preference for binding to the CCAAT boxes and to the enhancer region of several viral promoters (37, 45). High levels of C/EBP expression were found in terminally differentiated cells, e.g., hepatocytes and adipocytes (46), and it has been suggested that C/EBP plays a critical role in energy

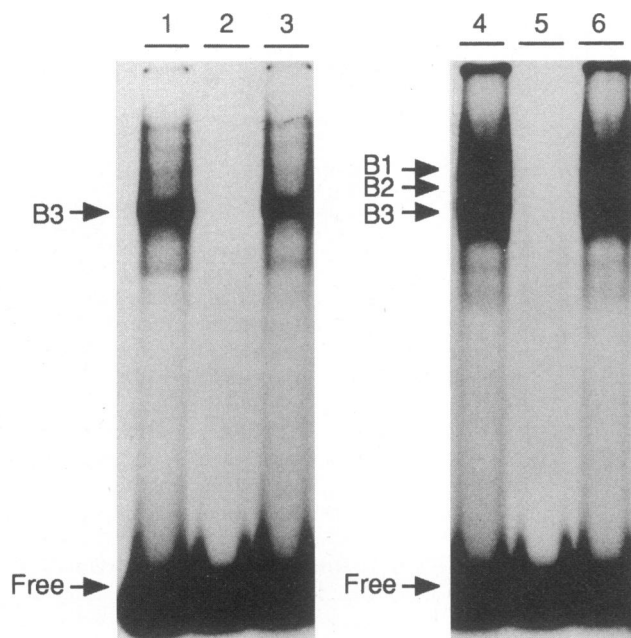


Figure 10. Gel-shift assay for C/EBP binding site of the PDGFR- α promoter. A 28-bp double-stranded oligonucleotide containing an enhancer core sequence for C/EBP was end labeled and incubated with 2 μ g of nuclear extracts from WKY- (lanes 1–3) or SHR-VSMC (lanes 4–6). For the competition experiments, a 200-fold molar excess of unlabeled oligonucleotides (lanes 2 and 5, C/EBP, and lanes 3 and 6, CTF/NF-1) was added and incubated on ice for 15 min before adding the labeled C/EBP probe. After incubation for 30 min at room temperature, reaction mixtures were resolved on a 4% polyacrylamide gel under nondenaturing conditions at 4°C in a low-ionic strength buffer before autoradiography. The positions of specific DNA–protein complexes are indicated as B1, B2, and B3 and that of the free DNA probe as Free.

metabolism, particularly in the synthesis and mobilization of glycogen and fat (47–49). Most recently, C/EBP has been identified as the adipocyte differentiation-related nuclear factor that activates the transcription of tissue-specific genes during the differentiation of 3T3-L1 cells (47, 50, 51). Our observations presented herein also suggest that C/EBP is an important *trans*-acting factor for the strain or cell-type specific transcription of the PDGFR- α gene in VSMC. However, the C/EBP family consists of multiple members that act as transcriptional activators (e.g., C/EBP α , LAP, C/EBP δ , and C/EBP γ) or transcriptional inhibitors (e.g., LIP and CHOP-10) and may control the transcriptional activity of the tissue-specific promoter by complex mutually inactive mechanisms that have not yet been clarified. Further studies will be needed to determine the detailed mechanisms of strain or tissue-specific transcriptional control in the PDGFR- α promoter.

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