INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

Platelet-Derived Growth Factor-Specific Regulation of the *MCP-1* Promoter in Rat Aortic Smooth Muscle Cells

by

Vladimir Y. Bogdanov

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

1999

This manuscript has been read and accepted by the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

1/25/99
Date

Mary R. Rifkin, Ph.D.
Chair of Examining Committee

1/26/99
Date

Terry A. Krulwich, Ph.D.
Executive Officer

Edward Fisher, M.D., Ph.D.

Ashok Kumar, Ph.D.

Jonathan Licht, M.D.

Heide Stuhlmann, Ph.D.

Supervisory Committee

The City University of New York

Abstract

Platelet-Derived Growth Factor-Specific Regulation of the *MCP-1* Promoter in Rat Aortic Smooth Muscle Cells

by
Vladimir Y. Bogdanov

Advisor: Professor Mark B. Taubman

Monocyte chemoattractant protein-1 (MCP-1) is a member of the family of "immediate early" genes induced by growth factors and cytokines in a variety of cell types. *MCP-1* encodes a low molecular weight secretory glycoprotein that is a member of the C-C subfamily of chemokines. MCP-1 is thought to play an important role in inflammation and in the recruitment of monocytes/macrophages to the vessel wall during the development of atherosclerosis and in vessel reocclusion following percutaneous transluminal coronary angioplasty (PTCA). Taubman et al. (1992) have previously reported that the induction of *MCP-1* in rat aortic vascular smooth muscle cells (VSMC) was specific for platelet-derived growth factor (PDGF) and was not seen with other growth agonists, including angiotensin II. This thesis describes the identification of a region in the proximal rat *MCP-1* promoter that is responsive to PDGF but not to other growth factors (angiotensin II and α-thrombin) or cytokines (interleukin 1β and tumor necrosis factor-α). Transient transfection assays of rat VSMC with promoter constructs

containing the luciferase reporter gene were the main experimental approaches used to identify the PDGF-responsive promoter region. The full response to PDGF (approximately 6 fold) requires the cooperative activity of two potentially novel *cis*-acting elements, at positions -146 to -128 and -84 to -59 of the published genomic sequence. These two elements produce different patterns in electrophoretic mobility shift assays. Nevertheless, they appear to bind the same PDGF-responsive protein species. Further analysis of these elements and trans-acting factors that interact with them should provide important insights into PDGF-specific responses in VSMC and may assist in developing new therapeutic approaches to highly selective inhibition of gene expression in the vessel wall.

Acknowledgements

Claire-Lise Rosenfield - for being my caring, responsive, and understanding lab mother. I thank her for teaching me all of the basic techniques and the encouragement to work when things were... a little slow.

Mark Taubman - for providing me with inspiration and support, for his ability and willingness to teach, and for being a much-needed role model. Unlike many a graduate student, I will never be able to relate to any of "preceptor horror stories" we have all heard on numerous occasions.

Andrea Weintraub - for being a good friend, a supportive lab neighbor and, to put it short, a fun pediatrician to hang out with.

Natalia Bogdanova - for being a loving, supporting and intelligent mother on whom I could always count and whose love was, and always will be, one of my most treasured possessions.

And, last but not least, a **few significant others** who made my years in graduate school so much more enjoyable. Some of them would not like to be mentioned together with the others, and for that reason they shall all remain unnamed. But when (if ever) they flip through this dissertation, they will know that I thought about each and every one of them with warmth and gratitude.

Table of Contents

List of Tables and Figures

Tables

Figures

Chapter 1: Introduction

Part 1: Monocyte Chemoattractant Protein-1 and its Role in Atherosclerosis

JE/MCP-1 (monocyte chemoattractant protein-1) is a member of the family of "immediate-early" genes (Herschman 1991) induced by growth factors in various cell types, including macrophages, endothelial cells, fibroblasts and vascular smooth muscle cells (VSMC) (Introna et al 1987; Rollins et al 1988; Rollins et al 1990; Shyy et al 1990; Taubman et al 1992; Yu et al 1990). The murine *MCP-1* analogue, originally designated as "*JE*," has been identified as a gene induced by platelet-derived growth factor (PDGF) in fibroblasts (Rollins et al 1988). The MCP-1 product is a secretory glycoprotein of the chemokine-β (or C-C) subfamily (Rollins 1991) whose members possess two contiguous cysteines near the amino terminus. The molecular size of murine MCP-1 is approximately 25 kDa (Ernst et al 1994), whereas human MCP-1 is significantly smaller, 13-15 kDa (Yoshimura et al 1989). MCP-1 attracts monocytes in vitro and in vivo but is not a chemotactic agent for neutrophils (Rollins 1991). MCP-1 also attracts CD4+ and CD8+ lymphocytes, basophils and natural killer cells in vitro and in vivo (Gunn et al 1997; Loetscher et al 1994). Human MCP-1 is a 76 amino acid protein with a complex three-dimensional structure: a long, flexible N-terminus followed by three antiparallel β sheets and a C-terminal α-helix (Steitz et al 1998). The N-terminal residues of the molecule, particularly an intercysteine first loop encompassing amino acids 13-35, are important for receptor binding and chemotaxis (Steitz et al 1998). The β sheet region of MCP-1 is important for its activity (Gong and Clark-Lewis 1995;

Zhang et al 1994). The functional role of the carboxy-terminal tail of MCP-1 remains uncertain; however, it is thought to be important for full chemoattractant potency (Valente et al 1991; Zhang et al 1994). Although MCP-1 binds to two 7-transmembrane segment G-protein coupled receptors, CCR2 and CCR9, it appears to signal only through the CCR2 receptor (Charo et al 1994; Nibbs et al 1997). There is some evidence that MCP-1 may exert its chemotactic activities in vivo in the form of a homodimer (Zhang and Rollins 1995).

Regulation of *MCP-1* expression in endothelial and vascular smooth muscle cells has been vigorously pursued due to the potential role of MCP-1 in the progression of atherosclerosis. MCP-1 protein has been identified in early human atherosclerotic lesions (Gerrity 1981; Takeya et al 1993) and has also been found in smooth muscle cells and macrophages of advanced human, primate and rabbit atherosclerotic plaques (Nelken et al 1991; Owens 1989; Wilcox 1992; Yla-Herttuala et al 1991; Yu et al 1992). In human plaques, MCP-1 has been identified in macrophages, VSMC and mesenchymal intimal cells (Nelken et al 1991). MCP-1 secreted by VSMC is likely to play a role in monocyte infiltration in the early development of the plaque. A possible positive feedback mechanism may feature infiltrating monocytes that secrete MCP-1 in large amounts, thus attracting more peripheral blood monocytes to the developing plaque (Nelken et al 1991). Inside the plaque, monocytes/macrophages transform into lipid-rich foam cells which confer instability to the structure of the plaque and thus increase the possibility of plaque rupture (Ross 1993). MCP-1 also upregulates expression of tissue factor in monocytes (Ernofsson and Siegbahn 1996) and VSMC (Schecter et al 1997). Elevated expression of tissue factor inside the

plaque is likely to promote thrombus formation, leading to the occlusion of the vessel. MCP-1-deficient mice exhibit severe defects in monocyte recruitment to sites of inflammatory damage. In a recent report (Gu et al 1998), mice deficient in MCP-1 and low density lipoprotein receptor were shown to have 83% less lipid deposition and many fewer monocytes in their aortas in comparison to control MCP-$1^{-/-}$ mice, thus corroborating the critical role of MCP-1 in the initiation and propagation of atherosclerosis.

Arterial restenosis, a common complication of percutaneous transluminal coronary angioplasty (PTCA), represents a special case of atherosclerotic progression. In the course of this invasive procedure, the vessel wall is exposed to many circulating growth agonists, including PDGF. Exposure of VSMC to these growth agonists leads to changes in phenotype and gene expression. VSMC are thought to be the primary source of MCP-1 at sites of acute vessel injury (Ross 1993). Increased *MCP-1* expression is likely to play a role in the pathophysiology of restenosis. A cascade model of restenosis has been proposed, whereby acute local thrombosis, blood coagulation and mechanical injury caused by PTCA activate cytokine gene expression by VSMC and macrophages within the plaque (Libby et al 1992). This leads to sustained autocrine and paracrine secretion of growth factors (i.e. PDGF, tumor necrosis factor α - TNFα, transforming growth factor β - TGFβ, and fibroblast growth factor - FGF) which causes activation of VSMC into proliferating, migrating and collagen-producing cells comprising the bulk of the occlusive neointima. Growth and migration of VSMC are though to be critical events in the pathogenesis of atherosclerosis, hypertension, angiogenesis and vessel reocclusion (Ross 1993).

A study conducted in Dr. Mark Taubman's laboratory established that, in VSMC, induction of monocyte chemotactic activity is highly specific for PDGF, in that agonists implicated in the development and progression of atherosclerosis, such as angiotensin II (Ang II) and FGF, as well as cytokines TNFα and interleukin 1-β (IL-1β) were unable to induce monocyte chemotactic activity (Poon et al 1996). PDGF did not act as a direct monocyte chemoattractant when placed in the modified Boyden chamber. Although numerous agents have been shown to have monocyte chemotactic activity, MCP-1 appears to account for all of the monocyte chemotactic activity secreted by cultured rat VSMC: the ability to induce monocyte migration was completely blocked by antisense oligonucleotides as well as antibodies to MCP-1 (Poon et al 1996). Interestingly, similar results were obtained in a study involving human endothelial and smooth muscle cells: antibodies to MCP-1 completely inhibited the monocyte chemotactic activity secreted by cells treated with minimally modified low density lipoprotein (Cushing et al 1990).

In summary, upregulation of *MCP-1* by PDGF at the site of vessel injury is likely to play an important role in VSMC-mediated monocyte recruitment during the development of atherosclerosis. Thus, the primary aim of my project is to identify a *cis*-acting element(s) in the *MCP-1* promoter conferring PDGF responsiveness in VSMC.

Part 2: *MCP-1* as a Model for Novel PDGF Signaling Pathways in VSMC

PDGF signaling has been intensely studied by many investigators. PGDF is a disulfide-linked dimer comprised of two homologous peptides (A and B) encoded by two distinct genes (Betsholtz et al 1986; Ross et al 1986; Waterfield et al 1983; Williams 1989). Three PDGF dimeric species, AA, AB and BB, are present in nature. The predominance of a particular PDGF isoform appears to be species and/or cell type specific; all three isoforms are mitogenic (Ross et al 1986). PDGF binds two monomeric units of the PDGF receptor, PDGFR-α or PDGFR-β (Ross et al 1986). The type of PDGF dimeric ligand determines the composition of the receptor dimer which is formed upon association with the ligand: PDGF AA induces formation of PDGFR-α homodimers, PDGF AB - PDGFR-α homodimers and α/β heterodimers, and PDGF BB - PDGFR-α and -β homodimers as well as α/β heterodimers (PJ Parker 1996). In VSMC, PDGFR-α and -β signal via distinct mechanisms, in that the α receptor primarily promotes cellular hypertrophy, whereas the β receptor is a more potent mitogen (Inui et al 1994). The extracellular domain of the PDGF receptor contains five immunoglobulin-like repeats. The cytoplasmic region has a tyrosine kinase domain interrupted by a kinase insertion sequence (Seedorf 1995). Upon dimerization, the PDGF receptor undergoes autophosphorylation, which creates binding sites for several SH-2 domain-containing proteins (e.g. phospholipase Cγ - PLCγ, phosphoinositol 3 kinase - PI3, Ras GAP, Shc) and thus leads to activation of a variety of intracellular signaling cascades. Following receptor stimulation, a number of serine/threonine kinases are activated, including c-raf, casein kinase II, mitogen-activating protein (MAP) kinase, and p44 MAPK2/ERK-1, which are likely to operate as components of the ras-regulated phosphorylation cascade (Kawahara et al

1994). Mutation analysis has shown that five tyrosine residues in the intracellular domain of the PDGF receptor at positions 740/751, 771, 1009 and 1021 are indispensable for binding PI3 kinase, Ras GAP, SHP-2, and PLCγ, respectively; tyrosine residues at positions 579/581, located in the juxtamembrane domain of the receptor, are required for Src binding (Montmayeur et al 1997). Recruitment of PLCγ results in activation of protein kinase C (PKC); interestingly, activation of protein kinase D by PDGF has been shown to require an intact tyrosine residue at position 1021 (a binding site for PLCγ) and occurs via activation of PKC (Van Lint et al 1998). The PDGF receptor has also been shown to bind and phosphorylate members of JAK and STAT families of transcription factors (Vignais et al 1996). MAP kinase and RSK S6 kinase have been identified as the two major downstream targets of PDGF signaling (Seedorf 1995). Activation of MAP kinase via PLCγ is *ras*-independent. PLCγ-induced hydrolysis of phosphoinositol phosphate 2 (PIP2) into the second messengers diacylglycerol (DAG) and IP3 results in mobilization of intracellular Ca^{2+}, a potent activator of many PKC isoforms (Seedorf 1995). MAP and RSK S6 kinases are involved in activation of a number of primary response genes upregulated by growth factors, such as *c-fos*, *c-jun* and *MCP-1* (Freter et al 1996; Herschman 1991; Marra et al 1993; Seedorf 1995; Takeshita et al 1995; Thiruvikraman et al 1996).

Members of Dr. Mark Taubman's laboratory have examined the expression of *MCP-1* mRNA and protein in rat aortic smooth muscle cells (Marmur et al 1992; Taubman et al 1992). *MCP-1*, *c-fos* and *KC*, an early-response gene which encodes a neutrophil chemoattractant, are rapidly induced by PDGF in vivo and in vitro (Marmur et al 1992). *KC* and *c-fos* expression is

also upregulated by Ang II; however, Ang II does not induce *MCP-1*. Interestingly, induction of *KC* and *c-fos* by PDGF in VSMC is blocked by downregulation of PKC by a 24-hour exposure to phorbol 12,13 dibutyrate, a PKC agonist. Induction of *KC* and *c-fos* by PDGF is also blocked by exposure of VSMC to BAPTA-AM and Quin 2-AM, chelators of intracellular calcium. Induction of *MCP-1* by PDGF is independent of activation of PKC or mobilization of intracellular calcium. The induction of *MCP-1* mRNA by PDGF is also independent of stimulation of the Na^+-H^+ exchanger, a signal shared by Ang II and PDGF and often involved in the induction of immediate early genes in VSMC (Taubman et al 1992). In addition, the PDGF-induced rise in intracellular cAMP is not involved in *MCP-1* induction by PDGF (Taubman et al 1992). Marumo et al. recently demonstrated that superoxide anion production in response to PDGF stimulation of human VSMC correlates with increased activity of NFkB as well as upregulated expression of *MCP-1* (Marumo et al 1997). Nevertheless, the precise events and signaling molecules involved in upregulation of *MCP-1* by PDGF in VSMC remain unknown.

Murine *MCP-1* was originally cloned from 3T3 fibroblasts stimulated with PDGF (Rollins et al 1988). Accordingly, a large proportion of data on *MCP-1* regulation by PDGF was obtained in murine fibroblasts. It was demonstrated that, in fibroblasts, PDGF induces *MCP-1* by a pathway distinct from the PDGF/serum pathway responsible for *c-fos* induction (Herschman 1991). This difference in intracellular signaling was thought to be the cause of a 60-90 min delay in *MCP-1* activation by PDGF, in contrast with a much more rapid upregulation of *c-fos* (Freter et al 1996; Geisterfer and Owens 1989; Hall et al 1989). Unlike the early-response genes *c-myc* and

c-fos, *MCP-1* is induced in 3T3 fibroblasts via a PKC-independent pathway (Hall and Stiles 1987). It should be pointed out that the mechanism of *MCP-1* induction by PDGF in VSMC appears to be PKC-independent (Marmur et al 1992). In mesangial cells, IL-1, TNFα, thrombin and interferon γ have been shown to induce *MCP-1* (Brown et al 1992; Grandaliano et al 1994a; Grandaliano et al 1994b; Rovin et al 1992); PDGF AB/BB and IL-1β induce *MCP-1* synergistically in this cell type (Goppelt-Struebe and Stroebel 1995). In a series of reports (Freter et al 1996; Freter et al 1995; Freter et al 1992), Freter et al. demonstrated the importance of NFkB signaling for induction of *MCP-1* by PDGF in murine fibroblasts. The role of one or more serine/threonine kinases in upregulation of *MCP-1* by PDGF in murine fibroblasts has also been established (Kawahara et al 1994). In summary, literature on *MCP-1* upregulation by PDGF in VSMC and other cell types is rather limited and does not contain detailed information about the intracellular events mediating *MCP-1* induction by this growth factor.

In contrast to other cell types, where the induction of *MCP-1* is common to a variety of growth agonists, the induction of *MCP-1* in VSMC is specific to PDGF and is not seen with other agonists, including Ang II or α-thrombin. Thus, upregulation of *MCP-1* expression by PDGF in VSMC, aside from being a critical event in propagation of atherosclerosis, appears to be an excellent model for studying signaling pathways unique to PDGF. The transcriptional component of *MCP-1* induction by PDGF may provide particularly important information about signaling pathways employed by PDGF but not by other growth agonists.

Part 3: *MCP-1* and Differences Between PDGF and Ang II Signaling in VSMC

VSMC exhibit two types of growth: hyperplasia, characterized by increased DNA and protein synthesis as well as cell division, and hypertrophy, characterized by increased cell size and protein content without DNA synthesis or cell division (Owens 1989). VSMC hyperplasia is an important feature of atherogenesis and is associated with the migration of VSMC from the vessel media to the intima and the proliferation of medial and intimal VSMC (Ross 1993). VSMC hypertrophy is more typical of the vascular response to chronic hypertension, although hyperplasia is seen in hypertension as well (Owens 1989). The growth responses of cultured VSMC appear to be agonist-specific. In adult rat aortic VSMC, PDGF and serum induce hyperplasia (Ross et al 1986). In the same cells, Ang II and α–thrombin induce hypertrophy (Berk et al 1989; Geisterfer and Owens 1989; Geisterfer et al 1988; Holycross et al 1993; Ross et al 1986; Rothman et al 1994; Schwartz et al 1990). Signaling events elicited in VSMC by PDGF and Ang II are likely to provide important insights into the similarities and differences between the programs of gene induction involved in the two types of growth.

An unusual feature of *MCP-1* regulation in VSMC is its responsiveness to PDGF but not Ang II. Ang II is a key component of the renin-angiotensin system, which regulates such physiologic responses as salt and water balance, blood pressure, and vascular tone. It is also a potent growth factor for VSMC that activates intracellular signaling events leading to induction of a number of genes, including *c-fos* (Berk and Corson 1997; Taubman et al 1989). Ang II is

produced in the vessel wall by renin and angiotensin converting enzyme (ACE). Renin converts angiotensinogen into angiotensin I, and ACE cleaves angiotensin I to create Ang II. Two receptors for Ang II, AT1 and AT2, have been identified. AT1 appears to be the predominant receptor isoform expressed in VSMC. AT1 is a member of the seven transmembrane-spanning, G protein-coupled receptor family. Although AT1 lacks intrinsic tyrosine kinase activity, many effects of AT1 stimulation require tyrosine phosphorylation and are shared by growth factor receptors, particularly PDGFR. G protein-coupled receptors stimulate PLC-β via G_q-mediated pathway (Berk and Corson 1997). Ang II also activates PLCγ through tyrosine phosphorylation (Berk and Corson 1997). AT1-mediated stimulation of PKC isoforms has been shown to upregulate MAPK activity via the Ras-Raf-MAPKK-MAPK cascade (van Bilsen 1997). Interestingly, Tyr 391 of AT1 is part of the Tyr-Ile-Pro-Pro motif, analogous to SH2 binding motifs found within PDGF receptors, i.e. Tyr-Ile-Ile-Pro (Berk and Corson 1997). This suggests that tyrosine phosphorylation of the AT1 receptor may be important in signal transduction events mediated by binding of SH2 domain-containing proteins (e.g. Src family kinases and PLCγ). Cross talk between G protein-coupled receptors and tyrosine kinase receptors is believed to include rapid activation of tyrosine kinase receptors by agonist binding to G protein-coupled receptors (Berk and Corson 1997). Stimulation of VSMC with Ang II results in tyrosine phosphorylation of Shc proteins, complex formation between Shc and an adaptor protein Grb2, and increased tyrosine phosphorylation of PDGFR-β that co-precipitates with with Shc-Grb2 complexes (Linseman et al 1995). These findings suggest that Ang II signal transduction may be in part mediated by ligand-independent tyrosine kinase receptor(s) activation (e.g. PDGFR and, possibly, receptors for EGF

and IGF-1). Another intriguing feature of Ang II receptor signaling involves interaction with cell matrix proteins comprising focal adhesion complexes (Berk and Corson 1997). Focal adhesion complexes play an important role in modulation of smooth muscle cell shape and volume and are critical in promoting migration, events mediated by Ang II. A 125-kD protein termed FAK is the best characterized tyrosine kinase localized to focal adhesion complexes (Kanner et al 1990). Ang II has been shown to stimulate tyrosine phosphorylation of FAK in VSMC (Okuda et al 1995; Polte et al 1994). A novel FAK homologue, CADTK, is stimulated by Ang II and PDGF in rat VSMC (Brinson et al 1998). For Ang II and PDGF, propagation of CADTK stimulation is dependent on cytosolic calcium signaling. Thus, focal adhesion molecules appear to be yet another point of convergence of signaling pathways employed by Ang II and PDGF in VSMC.

There is considerable literature involving the response of VSMC to PDGF and Ang (Bernstein and Berk 1993; Ross et al 1986; and references therein). Identification of signaling pathways unique to these two growth agonists remains an aim actively pursued by many groups of investigators. However, limited information on the differences between PDGF and Ang II signaling has been accumulated. The most likely reason for such slow progress is the fact that Ang II and PDGF stimulate many of the same intracellular signaling pathways, including the activation of phospholipase C, the induction of the MAP kinase cascade, and the activation of the Na^+-H^+ antiporter, and induce similar sets of early response genes (reviewed in Ross et al 1986 and Bernstein and Berk 1993). The effects of PDGF and Ang II on VSMC are summarized in Table1.

TABLE 1

COMPARISON OF EFFECTS ELICITED BY PDGF AND ANG II IN VSMC

EFFECT	PDGF	ANG II	REFERENCES
Hyperplasia	++	-	Ross, 1986
Hypertrophy	-	++	Berk et al., 1989
Activation of Na^+-H^+ exchanger	+	+	Taubman et al., 1992 Berk et al., 1987
Upregulation of protein kinase C	++	++	Marmur et al., 1992
Increase in intracellular Ca^{2+}	+	+	Ross, 1986; Marmur et al., 1992; Brock et al., 1985
Elevation of cAMP levels	+	-	Taubman et al., 1992
Induction of *KC*, *c-myc*, and *c-fos*	+	+	Taubman et al., 1989 Marmur et al., 1992 Taubman et al., 1992
Stimulation of FAK/CADTK	+	+	Brinson et al 1998
Induction of *MCP-1*	+	-	Taubman et al., 1992

Attempts by members of Dr. Taubman's laboratory to identify differences in gene expression in response to Ang II and PDGF using differential screening (Green et al 1995) or high resolution 2-dimensional protein gels (Patton et al 1995) have been unsuccessful, underscoring the similarities in signaling and gene induction between the two agonists. In a differential screening of over 1 million clones, only lysyl oxidase, thrombospondin, osteopontin and lactate dehydrogenase genes were found to be induced in VSMC by a 3-5 hour exposure of quiescent cultures to PDGF BB (Green et al 1995). All these genes were also induced by Ang II. The four proteins involved in protein folding, heat shock proteins HSP-60 and HSP-70, protein disulfide isomerase, and protein disulfide isomerase isozyme Q-2, were induced in quiescent VSMC in response to 24 hour treatment with PDGF and, notably, Ang II (Patton et al 1995). These studies suggest that there are a limited number of molecular events that distinguish the hypertrophic and hyperplastic responses of VSMC. The rat *MCP-1* gene may thus serve as an appropriate model system for identifying potential pathways that differentiate the response to Ang II and PDGF. Such pathways may be involved in differentiating the hypertrophic and hyperplastic VSMC phenotypes. Delineation of a PDGF-specific pathway in VSMC may be important in the therapy of atherosclerosis and in prevention of neointimal formation in response to balloon injury. Antibodies to PDGF (Ferns et al 1991) and a combination of antibodies to PDGF-BB and basic FGF (Rutherford et al 1997) drastically reduced intimal thickness and the intimal/medial area ratio in the rat model of balloon arterial injury. This suggests that PDGF BB is one of the major factors controlling neointimal hyperplasia. In a separate study (Sirois et al 1997), suppression of arterial

thickening was achieved by local administration of antisense oligonucleotides complementary to PDGF-β mRNA. This further supports the significance of PDGF receptor signaling for propagation of intimal hyperplasia. Selective inhibition of PDGF signaling in VSMC may thus provide valuable tools for modulation of cellular events in the vessel wall in a variety of pathophysiologic conditions. Identification of PDGF-specific *cis*-acting elements in the promoter region of the PDGF-responsive gene *MCP-1* may help bring this task closer to fruition.

Part 4: Previous Work on *MCP-1* Promoter Regulation

Rat, mouse and human *MCP-1* genes have been cloned and characterized (Rollins et al 1988; Rollins et al 1989; Timmers et al 1990). The rat *MCP-1* gene consists of three exons (151, 118 and 521 basepairs, respectively); the two introns are 680 and 330 basepairs. The predicted length of the rat MCP-1 product is 148 residues. The structure of the rat *MCP-1* gene is provided in Figure 1.

Regulatory elements in the promoter regions of *MCP-1* genes have been identified by several groups of investigators (Huang et al 1992; Li and Kolattukudy 1994; Tekamp-Olson et al 1990; Timmers et al 1990). A summary of functional *cis*-acting regulatory elements found in rat, murine and human *MCP-1* promoters is provided in Table 2.

TABLE 2

FUNCTIONAL *CIS* ACTING ELEMENTS FOUND IN RAT, MURINE AND HUMAN *MCP-1* PROMOTERS

SPECIES	ELEMENT (5'-3', UPPER STRAND)	POSITION RELATIVE TO START SITE	INDUCING AGENT AND/OR EFFECT	REFERENCES
Rat	TGACTCC	-52/-46	required for basal promoter activity	Timmers et al., 1990
Mouse	I. GGGCCTTTCCCTTGGCTGCTCCCAAG	-2537/-2512	PDGF	Freter et al., 1995
	II. CCTTTGTTGAGTCATTTCAGATTCTCC	-2494/-2468	PDGF	
	III. AGAACTGCTTGGCTGCAGGCCCAGCATC	-2457/-2430	PDGF	
	IV. AGAATGGGAATTTCCACGCTCT	-2319/-2298	PDGF	
Human	TCACTCA	-156/-150	TPA	Li et al., 1994
Human	TGACTCC	-128/-122	required for basal promoter activity	Li et al., 1994

Timmers et al (1990) have established that the -70/-38 region of the rat *MCP-1* promoter is necessary for its basal activity in transiently transfected 3T3 cells and that the region -141/-88 is essential for the response to the phorbol ester TPA. It should be noted that the -70/-38 region of the rat *MCP-1* promoter contains the sequence TGACTCC (-52/-46) related to the palindromic AP-1 consensus binding site (TGACTCA). The TATA box-like element AATAA is located at positions -30/-26. Li and Kolattukudy (1994) have established that two elements (at positions -156 to -150 and -128 to -123) in the promoter region of human *MCP-1* are necessary and sufficient to confer inducibility by TPA onto a CAT reporter construct transiently expressed in human glioblastoma cells. Activation of human *MCP-1* by TNFα occures via different *cis*-acting elements, because TNFα does not activate the TPA-inducible reporter constructs.

Freter et al (1992) identified two *cis*-acting regions that were necessary for the activation of the murine *MCP-1* gene by serum, PDGF, IL-1 and double-stranded RNA in transiently transfected BALB/c 3T3 fibroblasts. Attempts by Freter et al. to conduct promoter analysis using a reporter gene have been unsuccessful; thus, the study utilized RNAse protection assays with a series of tagged genomic *MCP-1* probes. The distal 240-bp sequence in the 5' flanking *MCP-1* region (positions -2537 to -2298 relative to the start site) possessed transcriptional enhancer activity. The other element identified in the study was a novel 7-mer, TTTTGTA, located in the 3' region of the murine *MCP-1* after the stop codon. Its activity was position dependent and orientation independent. The 7-mer is present in 3' regions of a number of transcription factors and cytokines belonging to the immediate-early gene class, including human

MCP-1. In follow-up studies (Freter et al., 1995, 1996), four PDGF-regulated elements were identified within the region located in the 240 bp 5' flanking murine *MCP-1* sequences. Two of these elements (I and IV) were similar in sequence and bound several forms of NFkB. The other two elements (II and III) were previously uncharacterized sequences which bound a PDGF-activated serine-threonine phosphoprotein found in the nucleus of PDGF-treated 3T3 fibroblasts.

The highly specific nature of *MCP-1* upregulation by PDGF in rat VSMC suggests that there may be PDGF-specific response element(s) in the rat *MCP-1* promoter. These putative elements are not among the previously identified PDGF-responsive elements because the sequence of the rat *MCP-1* promoter region does not contain any of the known PDGF-responsive sequences. These putative elements may therefore be part of a novel PDGF-specific pathway. Identification of these elements may provide tools to elucidate differences in signaling pathways activated by PDGF and Ang II.

Part 5: Summary and Project Aims

MCP-1 is a monocyte-specific chemokine whose elevated expression in smooth muscle cells of the vessel wall is a critical step in propagation of atherosclerosis and neointimal growth after PTCA. PDGF is thought to be a growth factor that is largely responsible for *MCP-1* upregulation in VSMC. Induction of *MCP-1* in VSMC is specific to PDGF and not other growth agonists, such as FGF, TNFα, IL-1β, or α-thrombin. In VSMC, this PDGF-specific response is observed with *MCP-1* and not with other immediate-early genes, i.e. *KC*, *c-fos* and *c-myc*. Most

importantly, induction of the rat *MCP-1* in VSMC does not occur with Ang II, a potent growth agonist which shares a large number of signaling pathways with PDGF. Signaling pathways induced by PDGF, but not Ang II, may be critical in differentiating hypertrophic and hyperplastic phenotypes exhibited by VSMC. These signaling pathways are likely to act upon a novel PDGF-specific response element in the rat *MCP-1* promoter because this promoter does not contain previously identified PDGF-responsive consensus sequences.

Dissection of the pathway employed by PDGF in upregulating *MCP-1* transcription in VSMC is likely to provide valuable tools for highly selective modification of MCP-1 activity in the vessel wall. Considering the important role MCP-1 protein plays in propagation of atherosclerosis and in development of post-PTCA vessel reocclusion, such tools may be of considerable clinical significance. Thus, the two major aims of my project are:

1. To identify a region in the rat *MCP-1* promoter that regulates its PDGF sensitivity in VSMC.

2. To identify and characterize a *cis*-acting element in the rat *MCP-1* promoter that differentiates the response to PDGF and Ang II.

Chapter 2: Materials and Methods

Growth Factors and Other Reagents

Recombinant human PDGF (BB, AA and AB isoforms) was obtained from Boehringer Mannheim Corporation (Indianapolis, IN). Recombinant murine IL-1β and TNFα were obtained from R&D Systems Inc. (Minneapolis, MN). Ang II was obtained from Sigma (St. Louis, MO). Purified α-thrombin was obtained from Dr. Peter Harpel (Mount Sinai School of Medicine, New York). Thermophilic DNA polymerase was purchased from Hoffman-La Roche (Nutley, NJ). TransCruz Gel Supershift reagents containing antibodies to c-Jun/AP-1 or Ets-1/Ets-2 (1.0 mg/ml) were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA).

Cell Culture

Smooth muscle cells were isolated from thoracic aortas of Sprague-Dawley rats by enzymatic dissociation as follows. 200-250 mg male animals (usually 6-8 weeks in age) were sacrificed and their aortas were removed from left subclavian origin to diaphragmatic insertion. Vessels were transfered into 100 tissue culture dishes with sterile Hank's balanced salt solution (HBSS). Under the microscope, adventitial fat was removed, after which aortas were transfered into 10 ml of sterile HBSS containing 175 U/ml of collagenase Type II (Worthington Biochemical corporation, Freehold, NJ) for 30 minutes. Following this treatment, aortas were washed with PBS, placed in fresh Dulbecco's modified Eagle's medium (DME) supplemented with 10% fetal bovine serum (Sigma) and 100 U/ml of penicillin/streptomycin (Gibco BRL, Gaithersburg, MD)

and left in a 37°C incubator with 5% CO_2 for 18-20 hours. A second, 2-hour digestion of aortas was then performed using HBSS-based solution containing 175 U/ml of collagenase Type II and 0.5 mg/ml og elastase (Sigma). Dissociated tissue was centrifuged for 5 minutes at 150x g, resuspended and grown in DME supplemented with 10% fetal bovine serum. To assess the purity of the obtained smooth muscle cells and rule out a significant fibroblast contamination, approximately 10^5 cells were plated in a 30 mm tissue culture dish and immunostained with monoclonal antibodies raised against human smooth muscle α-actin (Dako, Glostrum, Denmark). In subsequent passaging, cells were maintained in DME supplemented with 10% heat-inactivated calf serum (Sigma). Cells from spontaneously hypertensive rats (SHR) were prepared and grown in the same fashion; rats were 16 wks of age at the time of tissue harvesting. For transient transfection assays, cells were grown in 100 mm tissue culture dishes. For measurement of DNA or protein synthesis, VSMC were plated in 12 well culture dishes at 10^4 cells/well and incubated for 48 hrs in serum-free medium supplemented with bovine serum albumin (final concentration - 25 μg/ml), insulin (final concentration - 5.7 μg/ml) , holo-transferrin (final concentration - 5 μg/ml), and L-ascorbic acid (final concentration - 40 μg/ml). VSMC were then washed with phosphate-buffered saline (PBS) and incubated with growth agonists in the presence of [^{3}H] thymidine or [^{3}H] leucine (1 μCi/well) for 24 hrs. Cells were then fixed for 60 min at 4°C with 10% trichloroacetic acid (TCA). Incorporation of the radiolabeled material was determined by liquid scintillation spectrometry of the TCA-precipitable material as previously described (Schwartz et al 1990). Duplicate experiments were performed using 3 wells per treatment per experiment.

Isolation of RNA and Northern Blot Analysis

VSMC grown in 100 tissue culture dishes were washed with PBS and lysed in 4 ml/plate of 4M guanidine, 0.5% N-lauril sarcosine, 0.025M sodium citrate, pH 7.0. Chromosomal DNA was sheared by repeated passage of the lysate through a 21 gauge needle. 8 ml of lysate were then layered on to 3.5 ml of 5.7M CsCl, 0.1M EDTA and centrifuged at 10^5 g for 16 hours. The supernatant was removed by vacuum suction, and the RNA pellet was resuspended in 0.4 ml of diethyl pyrocarbonate (DEPC)-treated H_2O. The RNA was precipitated by the addition of 0.25M sodium acetate, pH 5.2 and 1 ml of of ethanol, and a 1 hr incubation at -20^{0}C. After centrifugation at 40C for 15 min at 10,000 g, the supernatant was discarded and the pellet was washed with 70% ethanol to remove contaminated salts. The RNA pellet was then resuspended by an overnight incubation in 0.25 ml of H_2O at 4^{0}C. The concentration of RNA was determined by the optical density at 260 nm. The pellet was reprecipitated and then resuspended in H_2O at a concentration of 2 μg/μl.

For Northern Blot analysis, 10 μg of total RNA was mixed with 3 volumes of loading buffer (final concentrations 48% formamide, 6% formaldehyde, 15 mM MOPS, 0.75 mM EDTA, 4% glycerol, 0.075% SDS, 0.075% xylene cyanol, and 0.075% bromphenol blue), heated to 65^{0}C for 2 minutes, and loaded onto an agarose gel (0.9% agarose, 10 mM MOPS, 0.5 mM EDTA, 3% formaldehyde, 100 ng/ml ethidium bromide). Following electrophoresis, formaldehyde was removed from the gel by two 30 minute washes in 10X SSC. Overnight transfer of the RNA to

nitrocellulose (Schleicher & Schuell) was performed using 10X SSC, and the filter was baked in a vacuum oven at 80^0C for 1 hour. A polaroid picture of the UV-illuminated filter was taken to verify equal amounts of ribosomal RNA in each lane. The filter was prewetted in 2X SSC and prehybridized overnight at 42^0C in 25 ml prehybridization buffer (5X SSC, 0.025M sodium phosphate, pH 6.5, 0.1% SDS, 250 mg/ml calf thymus DNA, 5X Denhardt's solution, 50% formamide), followed by overnight hybridization at 42^0C in 20 ml of hybridization buffer (5X SSC, 0.05M sodium phosphate, pH 6.5, 0.1% SDS, 100 mg/ml calf thymus DNA, 1X Denhardt's solution, 50% formamide) containing 30 X 10^6 cpm of ^{32}P-labeled cDNA probe. Final washes were performed using 0.5X SSC/0.1% SDS.

The full-length rat *MCP-1* cDNA insert used to generate ^{32}P-labeled probe was provided by Hong Zhang in 0.7% low-melt agarose gel. To radioactively label this DNA fragment, 25 µl of the gel was boiled for 5 minutes to denature the DNA and then placed on ice for 30 seconds. A 50 µl solution containing 40 mM each of dGTP, dTTP, and dATP, 2 mM ^{32}P-dCTP, 3.75 µg random hexamers, 67 mM KPO_4 (pH 7.5), 6.6 mM $MgCl_2$, 1 mM DTT, 10 units Klenow DNA polymerase, and 25 ng of DNA template was prepared. After a 1 hour incubation, unincorporated nucleotides were removed using a G-50 sephadex spin column.

Nuclear run-on assays

60-80% confluent VSMC were incubated in fresh DME +10% calf serum for 24 hrs. After treatment with PDGF BB for varying times, cells were washed with cold PBS, scraped into cold

PBS and centrifuged at 4^0 for 5 min at 500Xg. Cells were then resuspended in 4 ml of NP-40 lysis buffer (10 mM Tris HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40) and incubated on ice for 5 min followed by centrifugation at 500Xg for 5 min. The resultant pellet was again resuspended in 4 ml of NP-40 lysis buffer, incubated on ice for 5 min and centrifuged at 500Xg for 5 min at 4^0. Nuclear pellet was resuspended in 200 µl glycerol buffer (50 mM Tris HCl pH 8.3, 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA) and frozen at -70^0. Subsequently, nuclear pellets were resuspended in 400 µl TNE buffer (10 mM Tris HCl pH 8.0, 1 mM EDTA, 100 mM NaCl, 10 mM $MgCl_2$) and subjected to DNAse digestion (15 µl RQ DNAse I and 3 µl RNAsin, both obtained from Promega, Madison, WI) followed by Proteinase K digestion (42 µl 10% SDS and 16.5 µl Proteinase K at 20 mg/ml for 30 min at 37^0). Thus prepared RNA was phenol-extracted, ethanol-precipitated and used for run-on transcription assays using 1 ml of labeling mix comprised of 10 µl 1M Tris-HCl pH 8.0, 5 µl 1$MgCl_2$, 300 µl 1M KCl, 10 µl each of 100 mM ATP, CTP and GTP, 5 µl 0.25 mM UTP, 5 µl 1M DTT, 10 µl RNAsin and 100 µl ^{32}P-UTP (1,000 uCi). The resulting ^{32}P-UTP-radiolabeled RNA transcripts were hybridized to linearized and denatured full-length rat *MCP-1* cDNA immobilized on nitrocellulose filters using a Hybri-Dot blotter (Bethesda Research Laboratories, Gaithersburg, MD) under negative pressure with subsequent air-drying and baking for 1 hr at 80^0. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA and the pBluescript vector (Stratagene, La Jolla, CA) were used as positive and negative controls, respectively. Results were analyzed using a Phosphoimager (Molecular Dynamics, Sunnyville, CA). Levels of *MCP-1* transcript were normalized to those of the constitutively expressed

GAPDH after subtraction of background (pBluescript) and presented as fold increase over basal activity ($\pm$SEM). Studies were performed in triplicate.

Transient transfection assays

VSMC were plated at 10^6 cells/100 mm dish 24 hrs prior to transfections. Cells were co-transfected with 20 μg of *MCP-1* reporter constructs, 10 μg of human growth hormone expression vector (pXGH5, Promega, Madison, WI) and 10 μg of carrier DNA (pGEM4Z+, Promega). Transfections were carried out by $CaCl_2$ precipitation as follows. DNA mixtures were co-precipitated and resuspended in 500 μl of 0.25 M $CaCl_2$ and combined with 500 μl of 2X HEPES-buffered saline solution pH 7.12 (8.4 g NaCl, 5.9 g HEPES acid, 0.105 g Na_2HPO_4 in 500 ml) in a dropwise fashion with air being passed through the mixture using a mechanical pipettor. The precipitate was then evenly distributed over a plate of cells, after which chloroquine (final concentration of 10 mM) was added. Cells were exposed to the DNA/$CaCl_2$ mixture for 12-14 hours, washed with PBS and incubated in DME + 10% calf serum for 8 hrs. The medium was then switched to DME + 2% calf serum for an additional 38-42 hours. The medium was then collected and assayed for human growth hormone using the Growth hormone detection kit (Nichols Institute, San Juan Capistrano, CA). Fresh DME supplemented with growth factors was then added for 4-6 hours and protein extracts were collected and analyzed for luciferase activity (see below).

Plasmid construction

A 1093 bp fragment containing the 5' region of the rat *MCP-1* .gene (corresponding to bases -1040 to +40 of the published sequence [Timmers et al 1990] with an additional 13 bases at the 5' end) was cloned by Hong Zhang from a rat genomic library (Clontech, Palo Alto, CA) using a [^{32}P]αdCTP-labeled random primed probe generated from a full-length *MCP-1* cDNA template. This fragment was ligated by me into the luciferase reporter plasmid PXP2 (Nordeen 1988) to produce construct pJE-1053, whose sequence was confirmed by DNA sequence analysis using an Applied Biosystems DNA Sequencer, model 373A. Constructs with serial deletions at the 5' end of pJE-1053 were generated using the polymerase chain reaction (PCR) or restriction digestion and were named according to the number of base pairs they contained upstream of the start site previously established for the rat *MCP-1* gene (Timmers et al 1990). pGL-2 SV40 promoter vector was obtained from Promega (Madison, WI). All plasmid constructions were confirmed by DNA sequence analysis using an Applied Biosystems DNA Sequencer, model 373A.

Luciferase assay

Transiently transfected VSMC were washed twice at 25^{0}C with PBS, lysed for 15 minutes in 300μl/plate of Luciferase Cell Culture Lysis Reagent (Promega) and assayed for luciferase activity in a BioOrbit 1251 luminometer (Wallac, Gaithersburg, MD) using 20 μl of lysate and 100 μl of Luciferase Assay Reagent (Promega). Levels of luciferase activity were measured for 60 seconds and obtained numbers were normalized to levels of growth hormone detected in the

medium collected from the same plate prior to washing with PBS. The normalized luciferase activity detected in stimulated (i.e., growth agonist-treated) VSMC was expressed as fold induction ($\pm$SEM) over luciferase activity in unstimulated VSMC. Each condition was performed using duplicate plates and was repeated at least twice.

Electrophoretic mobility shift assay (EMSA)

VSMC were grown and treated as described for nuclear run-on studies. Preparation of nuclear protein extracts was carried out as follows. Approximately 10^6 cells were scraped into 2 ml of ice-cold PBS and pelleted for 1 min, after which the pellet was resuspended in 1 ml of cold hypotonic buffer (10 mM HEPES-KOH pH 7.9 at 4^0C, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.2 mM PMSF) by shaking tube. The cells were allowed to swell on ice for 15 min, and then vortexed for 15 seconds. Samples were then centrifuged for 15 seconds, and the supernatant fraction was discarded. The pellet was resuspended in 30-60 µl of cold extraction buffer (20 mM HEPES-KOH at 4^0C, 25% glycerol, 420 mM NaCl, 1.5 mM Mg Cl_2, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF) and incubated on ice for 20 min for high-salt extraction. Cellular debris was removed by centrifugation for 2 min at 4^0C and the supernatant fraction (containing DNA binding proteins) was stored at -70^0C. Total protein concentration in samples was determined using Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). Double-stranded DNA probes were end-labeled with [^{32}P]γATP (3000 Ci/mmol, NEN Research Products) using T4 polynucleotide kinase (New England Biolabs, Beverly, MA). 20-50,000 cpm (1-2 ng DNA) were incubated with 6 µg of nuclear extracts for 30 min on ice. To minimize

nonspecific DNA binding, 2 μg poly dIdC (Boehringer Mannheim, Indianapolis, IN) was added to each reaction. Binding reactions contained 20 mM HEPES pH 7.9, 12% glycerol, 1 mM $MgCl_2$, 1 mM EDTA, 1mM DTT, and 60 mM KCl. Unlabeled competitor DNA was added 10 min prior to the initiation of reactions. For EMSA supershift analysis, 4 μl of TransCruz Gel Supershift reagents (final concentration = 0.25 μg specific antibody /μl) were added to the samples together with the labeled probe. Protein-DNA complexes were resolved by electrophoresis on nondenaturing 4% polyacrylamide (40:1) at 4^0C in 50 mM Tris, 50 mM boric acid, 1 mM EDTA. Gels were transferred to polyacrylamide gel lift paper (Schleicher & Schuell), dried and analyzed by overnight autoradiography.

Chapter 3: PDGF BB Upregulates *MCP-1* Transcription in Rat Aortic VSMC

Optimization of Experimental Conditions for VSMC

MCP-1 is constitutively expressed in VSMC (Taubman et al 1992). Thus, by finding a cell culture condition associated with the lowest level of *MCP-1* expression, I expected to obtain the greatest increment in the response to PDGF. In order to determine such a condition, the following preliminary experiments were conducted. Rat VSMC were plated at 30-40% confluency, grown in DME supplemented with 10% calf serum for 24 hours, and then lysed. At the time of lysate collection, the medium in the remaining plates was switched to either fresh DME containing 0.3% calf serum or fresh DME containing 10% calf serum. Cells from each set were lysed every 24 hours. Total RNA was then isolated from cell lysates and analysed by Northern blotting using a ^{32}P-labeled *MCP-1* probe. The above experiment was performed 3 times, and the results obtained in each experiment were consistent. Figure 2 is a representative autoradiogram of a single experiment. The lowest level of *MCP-1* RNA in VSMC was obtained by incubating cells in 0.3% calf serum for 48 hours. This condition was thus chosen as an optimal pre-treatment of VSMC and subsequently utilized in nuclear run-on studies, transient transfections and EMSA analyses.

Nuclear Run-On Experiments

To verify that the accumulation of *MCP-1* mRNA in response to PDGF was due in part to stimulation of transcription, nuclear run-on assays were performed. Results of three nuclear run-on experiments are summarized in Figure 3. *MCP-1* transcripts were noted in unstimulated

VSMC. This finding is consistent with the presence of detectable levels of *MCP-1* mRNA by Northern Blot analysis. 20 ng/ml PDGF BB caused a 2.8±0.3-fold induction of *MCP-1* transcription at 30 min ($p<0.05$). *MCP-1* transcription returned to near baseline level (1.5±0.1, NS) within one hour.

Optimization of Transient Transfection Assays

To examine the regulation of the *MCP-1* promoter in rat VSMC, a 1093 bp fragment cloned in bacterial expression vector pGEM 4Zf+ (Promega, Madison, WI), consisting of bases -1040 to +40 of the published rat *MCP-1* genomic sequence with an additional 13 bp at the 5' end (Timmers et al 1990) was isolated by enzymatic digestion (Hind III/Bgl II), separated on a 1.5% agarose gel and ligated upstream of the luciferase gene in the PXP2 reporter vector. The resulting construct, pJE-1053, was then tested in transient transfections of VSMC. Four transient transfection experiments were carried out using pJE-1053/$CaCl_2$ mixture. In each experiment, 20 μg of pJE-1053 was used per 100 mm dish. Cells were incubated with DNA/$CaCl_2$ mixture for 18 hours, lysed, and cell lysates were then assayed for luciferase activity as described in Materials and Methods. No luciferase activity was detected in transfected VSMC (luminometer readings of 43±0.4, with background readings of 38-40). To verify that the procedure of DNA/$CaCl_2$ transient transfection was performed correctly, VSMC and COS cells were transfected with 20 μg of SV40-luciferase reporter construct (Promega, Madison, WI). The SV-40 promoter is constitutively active in most cell types. No luciferase activity was detected in VSMC lysates (luminometer readings of 38±0.5, n=2); COS lysates exhibited high levels of luciferase activity

(luminometer readings of 31,530±251, n=2). In conclusion, $CaCl_2$ precipitation appeared inadequate for transfecting rat aortic VSMC.

Two other methods of cell culture transfection, lipofection and electroporation, were then tested in VSMC. Four experiments were performed using the 20 μg pJE-1053 reporter construct and various amounts of lipofectamine, ranging from 40 to 180 μl per 100 mm dish. No luciferase activity was detected in lysates of transfected VSMC (luminometer readings of 44.3±0.1, n=4). Electroporation of VSMC was performed three times using 20 μg of pJE-1053 per 10^6 cells. The results of the electroporation were also negative - no luciferase activity was detected in lysates of electroporated cells (luminometer readings of 40.4±0.7, n=3). At that point, the decision was made to return to the DNA/$CaCl_2$ protocol and try to modify it in order to achieve significant and reproducible levels of luciferase activity.

The procedure of DNA/$CaCl_2$ transfection of VSMC was modified as follows: the amount of total DNA per 100 mm culture dish was raised to 40 μg (20 μg of pJE-1053, 10 μg of carrier DNA, i.e. pGEM 4Zf+, and 10 μg of pXGH5, a human growth hormone expression vector), and chloroquine was added along with the DNA/$CaCl_2$ mixture at a final concentration of 10 mM. In four experiments carried out according to the modified protocol, reproducible levels of pJE-1053 basal activity were obtained (luminometer readings of 608.5±32, n=4). Moreover, 10% CS and PDGF BB (20 ng/ml) induced pJE-1053 activity after a 4 hr treatment (87±4.5, n=2 in non-stimulated lysates vs 410.±23, n=2 in lysates of cells stimulated with PDGF). Experiments were

then conducted to determine the optimal time of cell lysate collection. As shown in Table 3, a 4 hr exposure to 10% CS resulted in an approximately 5 fold induction of pJE-1053 activity over levels in unstimulated transfected cells.

Based on the above, the protocol for subsequent transient transfections of VSMC was as follows: VSMC were transfected with 20 μg of a reporter construct along with 10 μg of carrier DNA and 10 μg of the human growth hormone expresstion vector pXGH5. Chloroquine was added to the DNA/$CaCl_2$ mixture at a final concentration of 10 mM. Transfected cells were incubated in low serum for 48 hours and subsequently treated with PDGF or other growth factors for 4-6 hours.

TABLE 3

CORRELATION OF LUCIFERASE ACTIVITY AND TIME OF EXPOSURE TO 10% CS

Duration of exposure to 10% CS	Luciferase activity (fold induction)
30 minutes	0.75
1 hour	1.2
2 hours	2.25
4 hours	4.85

The table shows results of a single representative experiment. Luciferase activity is normalized to levels of co-transfected human growth hormone. Fold induction is over levels obtained in non-stimulated transiently transfected VSMC.

PDGF-specific upregulation of the *MCP-1* promoter

Figure 4A shows upregulation of the transiently expressed pJE-1053 promoter construct by PDGF BB (20 ng/ml). Importantly, 1 μM Ang II and 1 μM α-thrombin failed to significantly increase *MCP-1* promoter activity. 10% CS and PDGF AB (20 ng/ml) had effects on *MCP-1* promoter activity similar to that shown for PDGF BB (Figure 4B).

To verify that the lack of responsiveness to growth agonists other than PDGF BB was not due to the absence of functionally coupled receptors, the effects of these agonists on VSMC growth were evaluated. As shown in Figure 5A, PDGF BB increased DNA synthesis by 820%±85% and 752%±224%, respectively. Typical of their hypertrophic action on adult rat aortic VSMC (Berk et al 1989; Geisterfer et al 1988; Patton et al 1995), Ang II and α-thrombin significantly increased protein synthesis (233%±15% and 257%±12%, respectively), but had no effect on DNA synthesis (Figure 5B). Of note, PDGF AA failed to increase DNA or protein synthesis, suggesting a low level of functionally coupled PDGF α-receptors in these cells (Poon et al 1996).

In addition to growth agonists, a variety of cytokines are also known to induce *MCP-1* in fibroblasts and endothelial cells (Hall et al 1989; Marra et al 1993). However, neither TNFα nor IL-1β induce monocyte chemotactic activity in the adult rat VSMC culture system used for these studies (Poon et al 1996). As shown in Figure 4A, 20 ng/ml IL-1β or 20 ng/ml TNFα failed to upregulate transcriptional activity of the JE-1053 reporter construct. Because IL-1β and TNFα do

not substantially alter DNA or protein synthesis in aortic VSMC, the activity of these agonists was verified by their ability to induce tissue factor: immunohistochemical staining of non-stimulated VSMC and VSMC treated with IL-1β and TNFα was performed by Veronica Gulle (laboratory of Dr. Jay Fallon, Mount Sinai School of Medicine).

PDGF induction of the *MCP-1* promoter is not affected by dexamethasone

A previous report from Dr. Taubman's laboratory (Poon et al 1991) demonstrated in vivo and in vitro inhibition of *MCP-1* expression by glucocorticoids in rat VSMC. This effect appeared largely due to rapid destabilization of *MCP-1* mRNA. To verify the lack of transcriptional component in the *MCP-1* inhibition by glucocorticoids, transient transfection assays using the pJE-1053 reporter construct were performed (Figure 6). The construct pJE-1053 lacks the glucocorticoid-sensitive region of *MCP-1* mRNA and thus should not be inhibited by dexamethasone. Indeed, addition of 10 μM dexamethasone to transfected cells along with PDGF did not affect luciferase activity, thus corroborating the absence of transcriptional component in the down-regulation of *MCP-1* expression by dexamethasone.

Chapter 4: Identification of two PDGF-specific response elements in the proximal *MCP-1* promoter

Sequence Analysis of the Proximal Rat *MCP-1* Promoter

The reporter construct pJE-1053 contains 1053 bp of the proximal rat *MCP-1* promoter and 40 bp of the 5'-untranslated region. The entire length of this genomic fragment was sequenced and found to be 100% identical to the published sequence (Timmers et al 1990). The sequence was then analysed for presence of known *cis*-acting elements using MatInspector software currently available online (genomatix.gsf.de). Four GATA-1 binding sites (positions -782, -602, -446, and -371) and two AP-1 consensus binding sites (positions -580 and -52) were identified. As noted in the Introduction, the AP-1 site at position -52 is located within the -70/-38 region of the rat *MCP-1* promoter that is necessary for its basal activity in transiently transfected 3T3 cells (Timmers et al 1990). Relative positions of the four GATA-1 and two AP-1 sites are shown in Figure 7.

Deletion Analysis

To locate the region(s) in the proximal *MCP-1* promoter that confers regulation by PDGF BB, constructs containing serial deletions from the 5' end of the *MCP-1* promoter were created using PCR and tested for luciferase activity. As shown in Figure 8, the response to PDGF remained unchanged for all deletions up to -146, indicating that the four GATA-1 consensus sequences and the AP-1 site at the position -580 were not involved in PDGF regulation of the

MCP-1 promoter. Deletion of bases -146 to -128 (corresponding to bases 894 to 912 of the published genomic sequence), containing the sequence TCCAAGGGCTCGGCACTTA (Element 1), resulted in an approximately 50% loss of PDGF stimulation. Deletion of the region between -128 and -84 did not additionally affect the PDGF response. Importantly, PDGF specificity was retained (see inset, Fig. 8A). Removal of bases -84 through -59 (corresponding to positions 956 to 981 of the genomic sequence), containing the sequence TGATGCTGCTCCTTGGCACCAACCAC (Element 2), eliminated most of the remaining response to PDGF, without changing the activity of the unstimulated promoter. Deletion of bases -59 through -42, containing the other AP-1 consensus binding site (TGACTCC), resulted in a complete loss of basal promoter activity.

Searches of the Genbank, EMBL and Eukaryotic Promoter databases were then conducted using sequences of Element 1, Element 2 and the entire region containing the two elements. No sequences homologous to the entire region containing both elements were identified; however, three regions highly homologous to Element 1 and a single region highly homologous to Element 2 were identified. The results of searches for sequences of both elements are shown in Figure 9.

Elements 1 and 2 are located 43 bp apart. To investigate the importance of this intervening sequence, Element 1 was ligated in the 5'-3' and 3'-5' orientations directly upstream of Element 2. As shown in Figure 10, these ligations [constructs pJE-84(1S) and pJE-84(1AS)] did not restore PDGF-induced *MCP-1* transcription to wild-type levels. Element 1 also failed to restore wild-

type levels of *MCP-1* transcription when placed in its proper position relative to Element 2, but in the opposite (3'-5') orientation [pJE-128(1AS)]. Thus, the cooperativity between Elements 1 and 2 may be positionally dependent and require the presence of at least some of the intervening 43 bases. Element 1 had no significant activity when placed directly in front of the AP-1 consensus binding site [pJE-59(1S)] in the absence of Element 2, suggesting that its proper position within the promoter is essential for its activity.

To determine whether Elements 1 and 2 exhibit enhancer activity, two luciferase reporter constructs, pGL(JE S) and PGL (JE AS), were generated and transiently expressed in VSMC. In these two constructs, a region of the *MCP-1* promoter corresponding to bases -146 to -59 was ligated directly upstream of the 195 bp of the proximal SV-40 promoter in the sense and antisense orientation, respectively. As shown in Figure 11, neither of the two constructs exhibited responsiveness to PDGF, suggesting that Elements 1 and 2 require a precise position and/or additional regulatory regions in the promoter sequence to confer activation by PDGF.

Attempts to establish a rat VSMC line with high response to PDGF

In order to pursue Aim 2, I decided to focus on Element 2 because this element alone was sufficient to elicit a PDGF-specific response in the rat *MCP-1* promoter. One major concern was the relatively low level of induction of construct pJE-84 which contained Element 2 but not Element 1. This could have posed a significant problem in obtaining statistically significant results while performing point mutation analyses or further deletions. Thus, a number of attempts were

made to establish new rat VSMC which might have a more potent response to PDGF. Three new VSMC strains were generated; each cell strain was originated from a pool of VSMC obtained from 6-8 Sprague-Dawley rats (200-250 mg). One strain became infected and was discarded. Two other cell strains (designated as VSMC 0196 and VSMC 0396) were tested in transient transfections using construct pJE-1053. VSMC 0196 exhibited a low response to PDGF BB (fold induction of pJE-1053: 3.72 ± 0.87, n=2), whereas VSMC 0396 exhibited a response to PDGF BB similar, but not higher than that of the VSMC strain that was being used in Dr. Taubman's laboratory at the time (fold induction of pJE-1053: 5.85 ± 1.62, n=2). I then attempted to establish a clonal VSMC strain with a high response to PDGF by using a dilutional cloning approach. A new rat VSMC strain, VSMC 0197, was established, and cells from this strain were plated in a 96-well tissue culture dish at a density of approximately 1 cell per well. Fourteen clones were obtained and expanded in 100 mm tissue culture dishes. All of the clones were tested by Dr. Michael Poon for their ability to induce monocyte chemotaxis in response to PDGF treatment. In his experiments, Dr. Poon used a Boyden chamber to assay for chemotactic activity of media samples collected from clones stimulated with PDGF BB. MCP-1 is the only monocyte chemotactic agent produced by cultured rat VSMC in response to PDGF BB, and it is thus reasonable to assume that an increase in chemotactic activity of the medium collected from stimulated VSMC would be proportionate to an increase in *MCP-1* expression and resulting increase in MCP-1 secretion. Only one clone (designated as 0197 C8) exhibited monocyte chemotactic activity higher than that of the previously established VSMC line. This clone was then tested in transient transfections using the construct pJE-1053. To my disappointment, 0197

C8 exhibited a very low response to PDGF BB (induction of pJE-1053: 1.73 ± 1.66, n=2). Based on these results, a decision was made to continue the analysis in the rat VSMC previously generated in the laboratory.

Point mutation analysis

To identify regions of Element 2 critical for PDGF regulation, a series of point mutations constructs (PMUT 1-3) were created; in each construct, three point mutations were introduced in Element 2. As shown in Figure 12, none of the point mutations significantly altered promoter activity, suggesting that a more substantial disruption of the wild type sequence might be necessary to identify functionally relevant regions. To achieve such a disruption, reporter constructs containing 7-8 bp cluster mutations in different regions of Element 2 in the context of a fully active *MCP-1* promoter (Figure 13, constructs A-D) were analyzed. All constructs had ≈50% of the activity of the wild type promoter, suggesting that the entire length of Element 2 is required for the wild-type response to PDGF. Complete mutation of Element 2 (construct E) also eliminated approximately 50% of full activity, demonstrating that in its appropriate context within the promoter, Element 1 retained activity. Construct E also failed to respond to Ang II (Figure 14), demonstrating that response of Element 1 was specific to PDGF.

To determine whether the response to Ang II and PDGF was unique to one type of VSMC, cells were also isolated from the aorta of SHR rats and used in transient transfection assays. VSMC isolated from SHR rats are known to have higher levels of Ang II receptors; thus,

absence of response to Ang II in these cells would give additional support to the notion that the rat *MCP-1* promoter indeed does not respond to Ang II in VSMC. Stimulation by PDGF induced similar levels of luciferase activity in these VSMC, whereas Ang II failed to elicit a response (Figure 14).

Chapter 5: Elements 1 and 2 bind multiple nuclear proteins

Optimization of EMSA conditions

To demonstrate that regions conveying PDGF-inducibility bind proteins, EMSA were performed using nuclear extracts from rat aortic VSMC. Initially, a 207 bp probe containing the PDGF-inducible region of the rat *MCP-1* promoter was tested in EMSA performed under standard conditions described in Current Protocols in Molecular Biology (Vol. 2, section 12.2). A representative result of this experiment is shown in Figure 15. Extracts from unstimulated and PDGF BB-stimulated VSMC produced three specific slow-migrating bands and one specific fast-migrating band. The lack of difference between the patterns obtained with unstimulated and PDGF BB-stimulated exctracts could be explained in a number of ways: 1) the probe was too large, bound too many protein species and thus could not detect slight differences between binding patterns; 2) the EMSA conditions were not optimal; and 3) no PDGF-inducible protein(s) interacted with Elements 1 and 2. To address the first possibility, two new EMSA probes were generated; these probes contained full sequences of either Element 1 or Element 2. A representative result of EMSA performed with Element 1 and Element 2 probes is shown in Figure 16. The Element 1 probe generated three specific slow-migrating bands and two specific fast-migrating bands, while the Element 2 probe generated a single specific slow-migrating band and two specific fast-migrating bands. Again, neither probe exhibited a pattern in PDGF BB-treated lanes distinct from that in unstimulated lanes. Consequently, a number of changes were made in EMSA conditions in order to maximize protein conservation and minimize disruption of

DNA-protein complexes. The concentration of the running buffer (TBE) was lowered from 0.5X to 0.25X, the EMSA were performed at 4^0C (instead of room temperature) and only freshly made nuclear exctracts were used. EMSA performed under the new conditions yielded results indicating the presence of a single PDGF-regulated protein fraction capable of binding to Element 1 and Element 2.

Elements 1 and 2 exhibit different binding patterns on EMSA

As shown in Figure 17A, a probe derived from Element 1 bound several protein fractions in a constitutive fashion. In addition, the binding of one fraction (marked by an arrow) was rapidly induced by PDGF. Binding of this fraction was not seen early after treatment with Ang II, but was noted 60 min after Ang II treatment. Element 2 also bound several protein fractions (Figure 17B). The slower mobility fraction was bound constitutively. However, binding of the faster moving species (marked by an arrow) was rapidly induced by PDGF, but not by Ang II. All fractions were successfully competed using a 300-fold molar excess of cold probe.

To further ascertain the relationship between the proteins binding to each element, competition experiments were performed. As shown in Figure 18A, an excess of cold Element 2 competed away the PDGF-regulated species binding to Element 1, but had little effect on the unregulated species. As expected, cold Element 1 competed away all binding. Similarly, Element 1 competed away the PDGF-regulated species binding to Element 2, but had no effect on the binding of the unregulated species (Figure 18B). These data suggest that both elements bind the

same PDGF-regulated protein. In addition, each element constitutively binds a distinct set of proteins.

Members of AP-1 and Ets families of transcription factors have been previously shown to upregulate *MCP-1* transcription in osteoblastic cells (Takeshita et al 1995) and respond to PDGF stimulation in VSMC (Naito et al 1998). The results of the sequence analysis of the proximal rat *MCP-1* promoter and the deletion analysis did not suggest that AP-1 or Ets proteins might be bound to PDGF-sensitive regions. However, certain members of these transcription factor families could possess an ability to bind to DNA sequences distinct from the established consensus binding sites. To investigate whether these proteins bind to either element, supershifts were performed using antibodies to c-Jun/AP-1 or Ets-1/Ets-2 (Figure 18A,B). No supershift bands or disruption of existing complexes were observed, suggesting that the bound complexes do not contain AP-1 or Ets proteins.

Chapter 6: Discussion

Previous studies from Dr. Taubman's laboratory have demonstrated that the accumulation of *MCP-1* mRNA and the induction of chemotactic activity in rat aortic VSMC is specific for PDGF and is not induced by a variety of other growth factors or cytokines known to be active in these cells (Poon et al 1996). This thesis describes the first analysis of the *MCP-1* promoter in VSMC and reports the identification of a PDGF-responsive area (-146 to -59) in the proximal rat *MCP-1* promoter. In addition, it demonstrates that this area has high specificity for PDGF and suggests that this may be responsible for the PDGF-specific accumulation of *MCP-1* mRNA seen in rat VSMC.

The full response to PDGF (approximately 6-fold) appears to require the cooperative activity of two *cis*-acting elements at positions -146 to -128 (Element 1) and -84 to -59 (Element 2). Either element alone is sufficient to confer PDGF-specific inducibility on a luciferase reporter construct, although the level of luciferase activity is approximately 50% of that seen with constructs containing both elements. Element 1 has no activity in the antisense orientation, when placed immediately upstream of Element 2, or when placed upstream of the AP-1-like site in the absence of Element 2. Its ability to confer full activity to Element 2-containing constructs thus appears to be dependent upon its position and orientation. It should also be stressed that the region of the rat *MCP-1* promoter containing Elements 1 and 2 is not capable of conferring PDGF

inducibility onto a heterologous promoter (i.e. SV-40), suggesting that proper positioning within the promoter and/or presence of additional regulatory elements may be an indispensable functional requirement for Elements 1 and 2.

Element 2 generates two major bands on EMSA. The amount of the slower moving species does not change significantly in response to PDGF, suggesting that the observed DNA-protein interaction is constitutive. In contrast, a faster moving species appears rapidly in response to PDGF, but not Ang II. As suggested by the deletion analyses, this species is likely to be of paramount importance in the PDGF-specific induction of *MCP-1*. Element 1 also binds several species, one of which is responsive to PDGF and appears to be the same PDGF-responsive species bound by Element 2. The observed binding of the PDGF-responsive species has a time course consistent with the nuclear run-on experiments. Of note, no early binding is seen in response to Ang II, but some binding is seen 1 hr after Ang II treatment. This later appearance may reflect secondary, rather than direct, effects of Ang II receptor stimulation and apparently is not sufficient for inducing the rat *MCP-1* promoter.

A model for PDGF upregulation of the rat *MCP-1* promoter in VSMC

The experimental results described in this thesis do not establish the precise boundaries of the PDGF-responsive elements. As shown in Figure 13, mutations in any region of Element 2 abolished this element's activity. This suggests that the entire sequence may be necessary for binding. Given the complexity of the binding patterns for both elements and the presence of

several species that are bound constitutively, it is highly likely that the PDGF effect requires the interaction of several proteins bound to different regions of the elements. The lack of effect of antibodies to c-Jun/AP-1 or Ets-1/Ets-2 on the binding of the PDGF-responsive species raises the possibility that the protein(s) involved may be different from those previously shown to mediate PDGF responses or to regulate *MCP-1* in other cell types. However, it is also possible that the protein complexes bound to Elements 1 and 2 contain AP-1 and Ets proteins whose epitopes are masked due to the configuration they maintain while bound to DNA. An experiment which would address this possibility is an EMSA performed with nuclear extracts depleted of AP-1 and Ets proteins with the help of specific antibodies. Lack of complex formation with such extracts would indicate that AP-1 and Ets transcription factors are indeed bound to Elements 1 and 2.

Figure 19 illustrates one possible model of upregulation of the rat *MCP-1* promoter by PDGF in VSMC. Protein species Y and Z are constitutively bound to Element 1 and 2, respectively. At the onset of stimulation by PDGF, protein species X (seen as a faster mobility shift band in EMSA) is rapidly activated (possibly via phosphorylation of tyrosine or serine/threonine residues) and targeted to Elements 1 and 2 in the rat *MCP-1* promoter. Fifteen minutes after PDGF stimulation, protein species X and Y are present on Element 1, whereas protein species X and Z are present on Element 2; at this stage, promoter activity is elevated approximately 6 fold. Two possible mechanisms are proposed to account for the formation of these complexes: protein-protein interaction between species X and species Y and Z or, alternatively, direct binding of X to both elements. Given the difference in the sequences of the

two elements, protein-protein interaction is more likely to account for the formation of X+Y and X+Z complexes. It should be stressed, however, that direct interaction between species X and DNA regions comprising Element 1 and Element 2 does occur even if species X is initially recruited to the site via protein-protein interaction: the results of EMSA demonstrate the ability of species X to bind probes containing Element 1 or Element 2. One hour following PDGF stimulation, species X is still present on Element 1, but not on Element 2, and the promoter activity reverts to baseline levels.

Sequence analysis of Element 1 and Element 2

Searches of the Genbank (last search: Release 90.0, 9/98), EMBL (last search: Release 45.0, 9/98) and Eukaryotic Promoter (last search: Release 50, 9/98) databases with both putative elements and the entire fragment containing both elements suggest that the sequences conveying PDGF specificity may be novel. As shown in Figure 9, Element 1 has high homology to a 5' flanking region of Mus musculus cathepsin B (accession number X76621) and to areas in the promoter regions of Aspergillus niger PX18 (accession number M90701), Homo sapiens gastrin (accession number EPD 25015) and Mus musculus keratin (accession number EPD 32005). Element 2 has high homology only with sequences in the 5' region of the murine *MIP-2* gene. MIP-2 protein is a cytokine with potent chemotactic activity for human polymorphonuclear leukocytes (Huang et al 1992; Tekamp-Olson et al 1990). None of the genes identified contain areas of high homology with both elements, and searches of the literature did not provide evidence that these genes were PDGF responsive. As shown in Figure 20, Element 1 and 2 are both

present in the murine *MCP-1* promoter; however, Element 1 has a significantly lower homology (79%) to the rat sequence than Element 2 (98%).

No regions highly homologous to either element could be detected in the human *MCP-1* gene, suggesting that these elements may be species specific. Only 526 bases of the human *MCP-1* promoter sequence has been cloned to date (Li and Kolattukudy 1994). There is thus a possibility that there are homologous elements in the human *MCP-1* gene located further upstream. It should also be noted that PDGF and MCP-1 have been co-localized in human atherosclerotic plaques (Nelken et al 1991; Wilcox 1992), which raises the possibility that MCP-1 production by human VSMC is increased in response to PDGF. However, regulation of human *MCP-1* by PDGF is yet to be described.

As was mentioned earlier, Freter et al. (1992, 1995, and 1996) have conducted a rigorous study of the murine *MCP-1* promoter in 3T3 fibroblasts. Interestingly, the differences between the murine and the rat *MCP-1* promoters appear to be rather broad: in the rat *MCP-1* gene, murine NFkB binding PDGF-responsive elements I and IV are not present. There is a region in the rat *MCP-1* promoter (positions -472 to -449) with a 72% homology to murine element II in reverse orientation (see Figure 20); however, my data indicate that the presence of this region is not required for activation of *MCP-1* in VSMC by PDGF. The rat *MCP-1* gene also possesses a region with 69.2% homology to murine element III in reverse orientation; this region is located 15 base pairs downstream of the initiation codon. Thus, the pathways which lead to transcriptional

induction by PDGF of *MCP-1* in mouse appear to be different from those in rat and may also be different in VSMC from other cell types.

Upregulation of *MCP-1* gene by PDGF in rat VSMC

Dr. Taubman's laboratory has previously reported that changes in mRNA stability were responsible for part of the increase in *MCP-1* mRNA levels seen after PDGF treatment (Taubman et al 1992). In that study, nuclear run-on experiments, performed at 1 and 2 hrs, did not suggest a major transcriptional component. These time points were originally chosen because of the delayed peak in *MCP-1* mRNA levels. My study employed considerably earlier time points (15 min and 30 min) to examine *MCP-1* transcription. Using these time points, PDGF was found to cause a marked, but very transient increase in *MCP-1* transcription. Taken together, these studies suggest the following. The initial response to PDGF is an elevation in transcription, which is short lived. This is accompanied by an increase in mRNA stability (half life of 2.5 hrs vs. 0.5 hrs), resulting in a prolonged elevation in *MCP-1* mRNA levels beyond that seen with other immediate-early genes. Figure 21 illustrates upregulation of *MCP-1* mRNA levels by PDGF in VSMC in the form of a graph.

As noted in the introduction, the intracellular signaling pathways responsible for *MCP-1* mRNA accumulation in rat aortic VSMC remain to be determined. Initial studies employing RNA blot analyses and a variety of inhibitors failed to demonstrate a role for protein kinase C, mobilization of intracellular calcium, activation of the Na^+-H^+ exchanger, and changes in cyclic

AMP (Taubman et al 1992). While these studies were by no means exhaustive and do not rule out participation of any of these signals, they do suggest that the induction of *MCP-1* mRNA in rat aortic VSMC by PDGF involves signaling pathways distinct from those involved in the induction of a number of other immediate early genes in rat VSMC, including c-*fos*, *KC*, and tissue factor (Freter et al 1996; Freter et al 1995; Taubman et al 1993). The studies described in this thesis, demonstrating potentially novel PDGF-specific elements in the rat *MCP-1* gene, give further credence to the concept that the regulation of *MCP-1* in rat aortic VSMC may involve an unusual set of signaling pathways.

Identification of transcription factors interacting with Elements 1 and 2: future directions

The next step in researching the mechanism of *MCP-1* upregulation by PDGF in VSMC must include identification of proteins that interact with Elements 1 and 2. The size of the protein often serves as an important step in identifying its nature. A simple experiment could provide information about the size of the protein complexes bound to Elements 1 and 2. Namely, radioactively labeled Element 1 and 2 probes could be incubated with nuclear extracts from PDGF-stimulated VSMC, UV-crosslinked to bound proteins, and run on an SDS-PAGE gel along with radioactively labeled protein markers. A gel autoradiogram would reveal the number and approximate size of proteins bound to each element.

Biochemical purification of these *trans*-acting factors appears to be another appropriate initial experiment. It should be pointed out that smooth muscle cell cultures are difficult to make

and maintain and virtually impossible to grow in large volumes. At the same time, purification of nuclear factors using a DNA binding site usually requires vast amounts of nuclear extracts. I would thus propose to investigate whether a similar pattern of PDGF induction of the rat *MCP-1* promoter can be obtained in different cell cultures that can be more easily expanded, e.g. 3T3 fibroblasts or HeLa cells. EMSA should be performed to verify that they produce similar bands, suggesting that the same nuclear proteins are responsible for upregulation of *MCP-1* transcription by PDGF. Then, a biotin-streptavidin purification system can be created in which biotin-labeled double-stranded oligonucleotide containing sequences of Element 1 or Element 2 is incubated with nuclear extracts of cells stimulated with PDGF. A variation of this method, called magnetic DNA affinity purification (Gabrielsen et al., 1993) seems particularly appropriate. According to this method, biotin-labeled DNA containing *cis*-acting elements of interest is attached to streptavidin-covered magnetic beads placed in a tube with a magnet underneath it. Protease inhibitors (e.g. DTT, PMSF) must be added to the incubation mixture to ensure stability of freshly extracted nuclear proteins. Incubation with nuclear extracts followed by addition of an excess of non-specific DNA competitor allows for the separation of proteins bound to DNA with high affinity from the rest of the proteins in the extract. After elution by high salt wash, these *trans*-acting factors are then used in EMSA to verify that they indeed bind to Elements 1 and 2. The amounts of the isolated protein should be sufficient to sequence the protein and thus determine its composition, secondary structure and possible relation to members of known transcription factor families.

The above described experiments are most likely to yield PDGF-regulated protein species that interact with Elements 1 and 2. Results of EMSA indicate, however, that the PDGF-regulated protein might be somewhat unstable in vitro and its interaction with DNA and other proteins bound to the site may be weak. Thus, an alternative experiment aimed at identification of the PDGF-regulated protein that interacts with Elements 1 and 2 would involve a VSMC line permanently transfected with a luciferase reporter construct containing both elements (e.g., pJE-146). A cDNA library made from PDGF-stimulated VSMC would then be used to transfect this cell line and screen for plates with induced luciferase activity. A pure clone encoding the PDGF-regulated transcription factor could be obtained after several rounds of enrichment for the cDNA of interest. The protein could then be expressed in a mammalian cell line (e.g., COS or CV1 cells), purified, and its ability to interact with Elements 1 and 2 verified by EMSA. The protein's cDNA sequence would allow for screening of a genomic library and, possibly, identification of a novel family of transcription factors.

Chapter 7: Significance

MCP-1 and PDGF are thought to play central roles in the development of atherosclerotic lesions. Early stages of atherosclerosis are characterized by endothelial cell disfunction, brought about by a variety of factors, including oxidized LDL. Disfunctional endothelial cells synthesize MCP-1, which leads to the recruitment of circulating monocytes to the vessel wall. Monocyte infiltration is a critical step in the development of the atherosclerotic plaque. Some of the recruited monocytes become foam cells and thus contribute to the thickening of the arterial wall. More importantly, recruited monocytes differentiate into macrophages and secrete growth factors, such as PDGF, that stimulate VSMC proliferation and thus contribute to the development of atherosclerotic plaques. Stumulated VSMC produce large amounts of MCP-1, thus stimulating monocytic invasion of the vessel wall and accelerating the developement of atherosclerosis.

In an injured vessel, stripped of endothelium (i.e. post-PTCA coronary artery), platelets and leukocytes aggregated at the site of injury release cytokines and growth factors, including PDGF. VSMC exposed to PDGF synthesize MCP-1, thus stimulating monocytic infiltration. Recruited monocytes synthesize PDGF and other growth factors, thus promoting intimal hyperplasia and, ultimately, vessel reocclusion.

A recent study conducted in Dr. Taubman's laboratory (Poon et al, 1998) demonstrated that treatment of hypercholesterolemic rabbits with trapidil, an inhibitor of PDGF signaling,

resulted in a 75% reduction in MCP-1 and macrophage accumulation in the arterial wall. This suggests that PDGF is a major stimulant of MCP-1 expression in the vessel wall, and also suggests that MCP-1 is responsible for marophage accumulation. In another recent study (Rogers et al 1998) it was demonstrated that blocking antibodies to the leukocyte integrin Mac-1 significantly decreased intimal thickening after angioplasty in a rabbit model. These data suggest that recruitment of white blood cells to the site of arterial injury is a major event in the development of intimal hyperplasia, and further suggests that MCP-1, whose expression in the vessel wall is largely controlled by PDGF, is one of the major chemokines responsible for the recruitment of monocytes to the site of arterial injury.

This thesis describes a mechanism of transcriptional upregulation of the *MCP-1* gene by PDGF. This mechanism may contribute to increased levels of MCP-1 seen in the atherosclerotic vessel wall. Further elucidation of intracellular pathways responsible for transcriptional upregulation of *MCP-1* by PDGF may provide valuable insights into the regulation of gene expression in atherosclerotic lesions and improve our understanding of intracellular signaling in VSMC.

Induction of *MCP-1* in cultured rat aortic VSMC is specific to PDGF and not other growth factors, such as Ang II. *MCP-1* may thus be an appropriate marker for studying PDGF-specific signaling pathways. This thesis describes a PDGF-specific response region in the proximal rat *MCP-1* promoter that contains two novel *cis*-acting elements. Identification of the *trans*-acting

factors that interact with these elements is likely to provide important insights into intracellular components of a novel PDGF-specific signaling pathway in VSMC.

PDGF and Ang II have been implicated in the development of atherosclerosis, in the response of the vessel wall to injury, and the vascular changes associated with hypertension (Owens 1989; Ross 1993; Taubman et al 1989). Although considerable information exists about the signals induced in VSMC by PDGF and Ang II, the induction of *MCP-1* remains one of the few phenomena described that distinguishes the early response of rat VSMC to these two agonists. MCP-1 protein appears to function chiefly as a monocyte chemoattractant and has not been shown to be a mitogen. It is therefore unlikely that MCP-1 is directly responsible for the difference between PDGF-induced hyperplasia and Ang II-induced hypertrophy. Nevertheless, identification of a PDGF-specific transcriptional mechanism involved in regulation of *MCP-1* expression in VSMC may prove to be an important discovery. This thesis describes two *cis*-acting elements in the rat *MCP-1* promoter that respond to PDGF but not Ang II. These elements may be useful in developing novel therapies aimed at inactivation of PDGF-induced genes. Targeting of specific signaling intermediates employed by PDGF rather than PDGF or its receptor might be a far more sophisticated and promising approach to modulating gene expression in VSMC. Such an approach might allow one to inactivate a small number of target genes while leaving the majority of PDGF-stimulated genes intact. Finally, identification of the proteins interacting with Elements 1 and 2 may provide new targets for modulating PDGF-specific events associated with atherosclerosis.

Chapter 8: Figures

Figure 1. Structure of the rat *MCP-1* gene

The flag indicates the cap site; A_n denotes the poly(A)-addition site. The three exons are indicated by boxes. The filled parts of the boxes represent the coding sequence.

Figure 1

Figure 2. Levels of *MCP-1* mRNA in VSMC grown under different conditions

Shown in the figure is a representative Northern Blot analysis of *MCP-1* mRNA expression in growing VSMC (lane 1), VSMC placed in DMEM supplemented with 10% calf serum and collected at 24, 48, 72 and 96 hours, respectively (lanes 2-5), and VSMC placed in DMEM supplemented with 0.3% calf serum and collected at 24, 48, 72 and 96 hours, respectively (lanes 6-9). The integrity of RNA used for analysis was confirmed by ethidium bromide staining of the 28S and 18S rRNA.

Figure 2

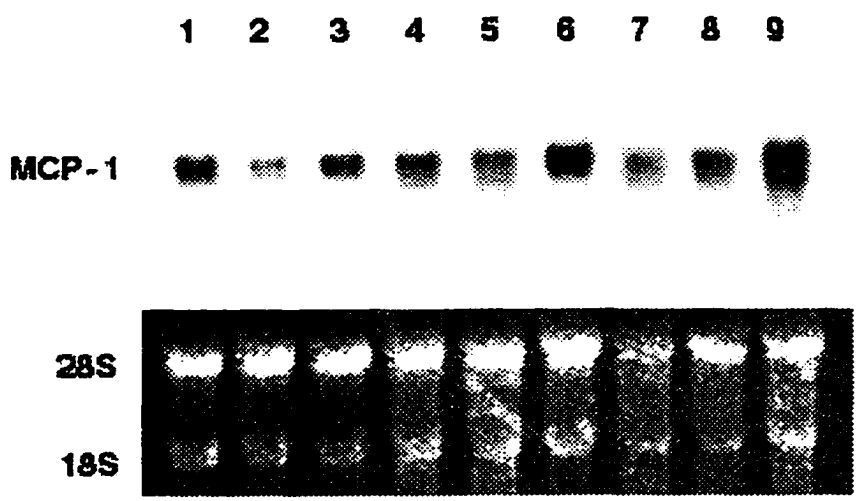


Figure 3. *MCP-1* transcription in rat aortic VSMC

Quiescent VSMC were stimulated with PDGF BB (20 ng/ml), nuclei were harvested at the times indicated, and nuclear run-on assays were performed. The figure is a graphic summary of all nuclear run-on experiments. Values are normalized to GAPDH and expressed as fold induction over levels in non-stimulated VSMC, error bars are ± SEM (n=3). **Inset:** a representative autoradiogram showing hybridization to pBluescript (pBS) DNA, rat JE/MCP-1 cDNA, and GAPDH cDNA.

Figure 3

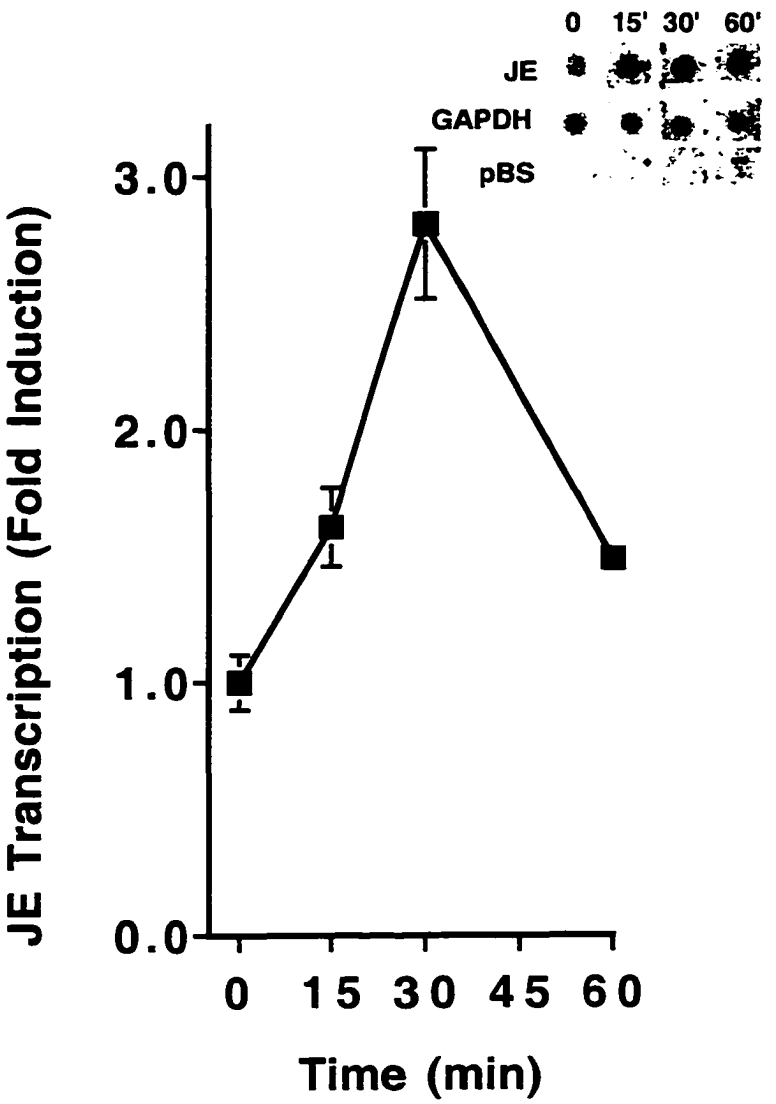


Figure 4. Induction of the *MCP-1* promoter in VSMC: agonist specificity

A) Subconfluent VSMC cultures were transiently transfected with the *MCP-1* luciferase reporter construct pJE-1053 and stimulated with PDGF BB (20 ng/ml), PDGF AA (20 ng/ml), Ang (1 μM), α-thrombin (1 μM), TNFα (20 ng/ml), and IL-1β (20 ng/ml). Luciferase activity, obtained in duplicate experiments, is normalized to co-transfected human growth hormone (GH) and expressed as fold induction over levels in non-stimulated (treated with fresh DME without growth factors) transiently transfected VSMC. Error bars are ± SEM.

B) Subconfluent VSMC cultures were transiently transfected with the *MCP-1* luciferase reporter construct pJE-1053 and stimulated with 10% calf serum (CS), PDGF BB (20 ng/ml), and PDGF AB (20 ng/ml). Luciferase activity is normalized to co-transfected human growth hormone (GH) and expressed as fold induction over levels in non-stimulated (treated with fresh DME without growth factors) transiently transfected VSMC. The graph represents results obtained in a single transfection experiment.

Figure 4A

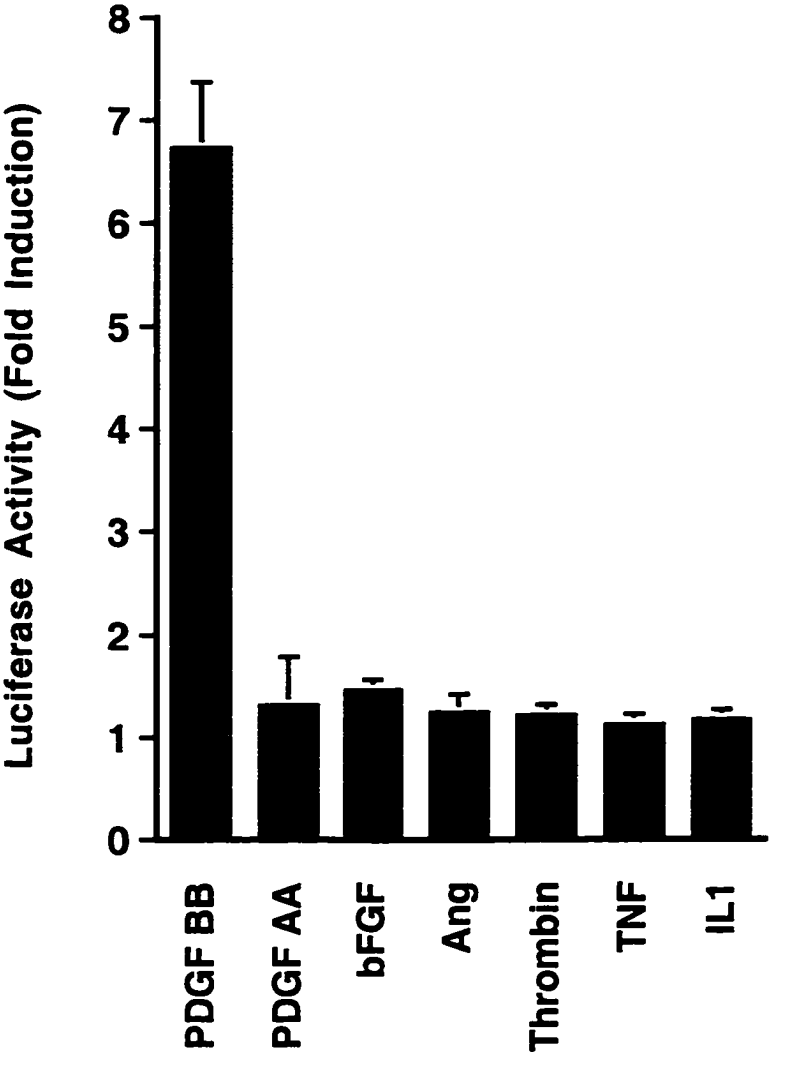


Figure 4B

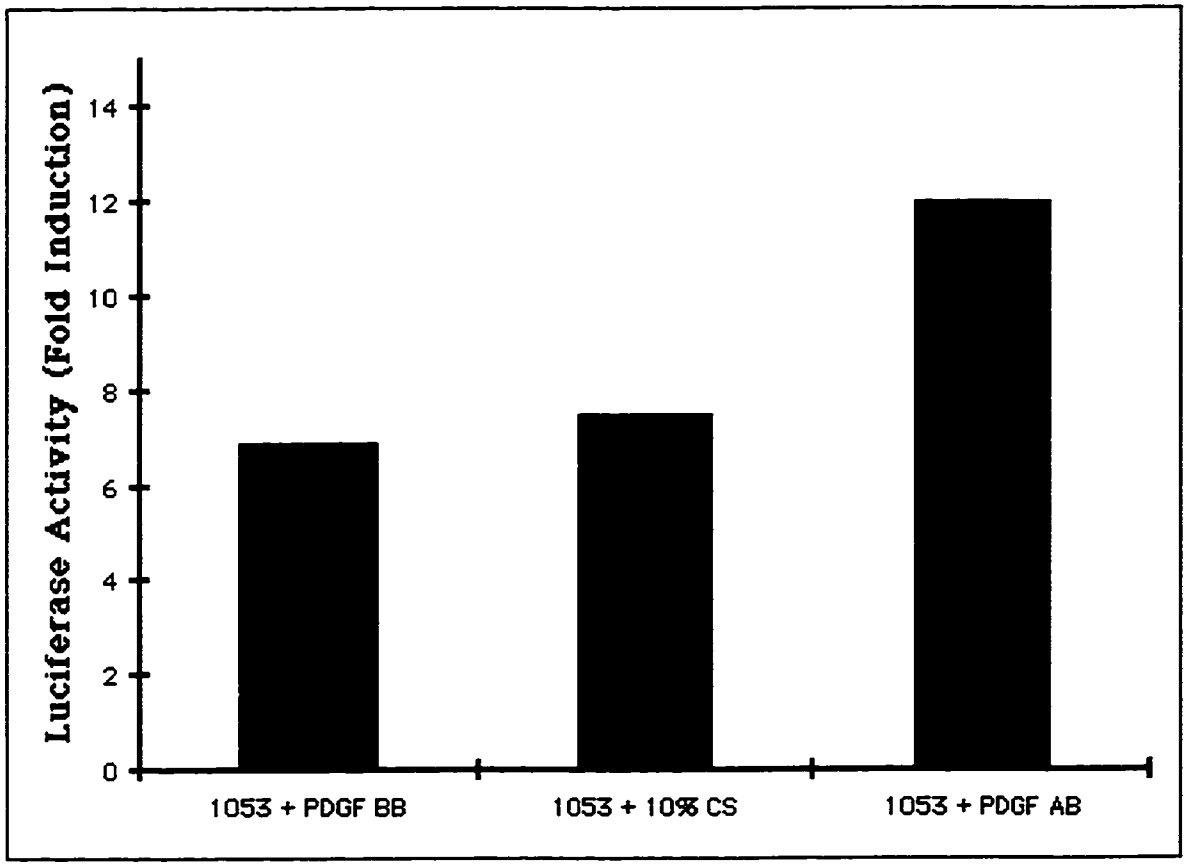


Figure 5. Effects of growth agonists on DNA and protein synthesis in VSMC

A) VSMC were incubated with growth agonists (concentrations as in Figure 2) in the presence of [^{3}H] thymidine (for DNA synthesis) or **B)** in the presence of [^{3}H] leucine (for protein synthesis) for 24 hrs. Incorporation of radiolabeled material was determined by scintillation spectrometry. Values are obtained from duplicate experiments employing triplicate wells and are expressed as percent of [^{3}H] incorporation relative to levels observed in non-stimulated VSMC. Error bars are ± SEM.

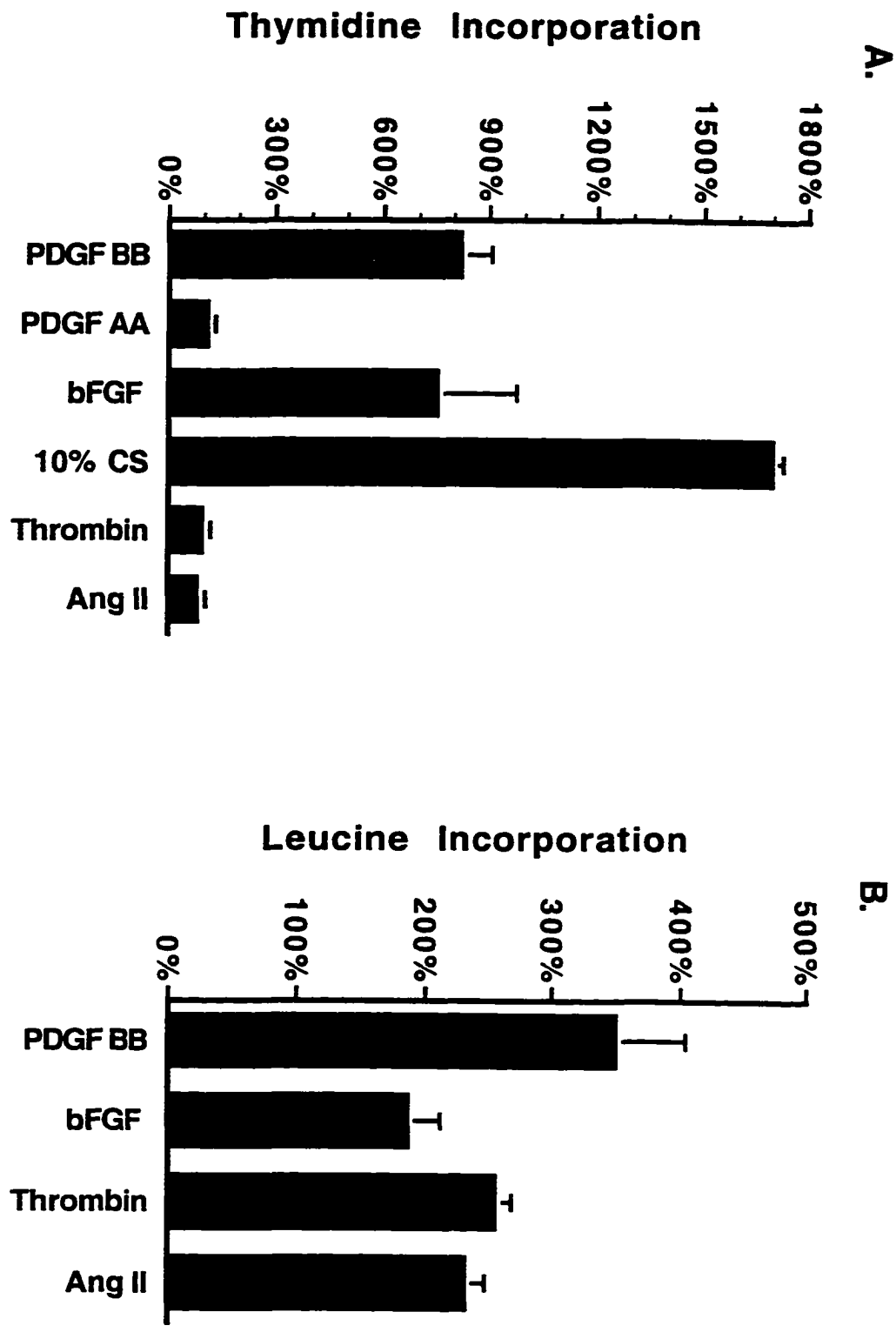


Figure 5

Figure 6. Effects of dexamethasone on *MCP-1* promoter

VSMC were transiently transfected with the reporter construct pJE-1053 and stimulated with either PDGF alone (20 ng/ml) or with PDGF+10 μM dexamethasone for 4-6 hours. Luciferase activity, obtained in triplicate experiments, is normalized to co-transfected human growth hormone (GH) and expressed as fold induction over levels in non-stimulated (treated with fresh DME without growth factors) transiently transfected VSMC. Error bars are ± SEM.

Figure 6

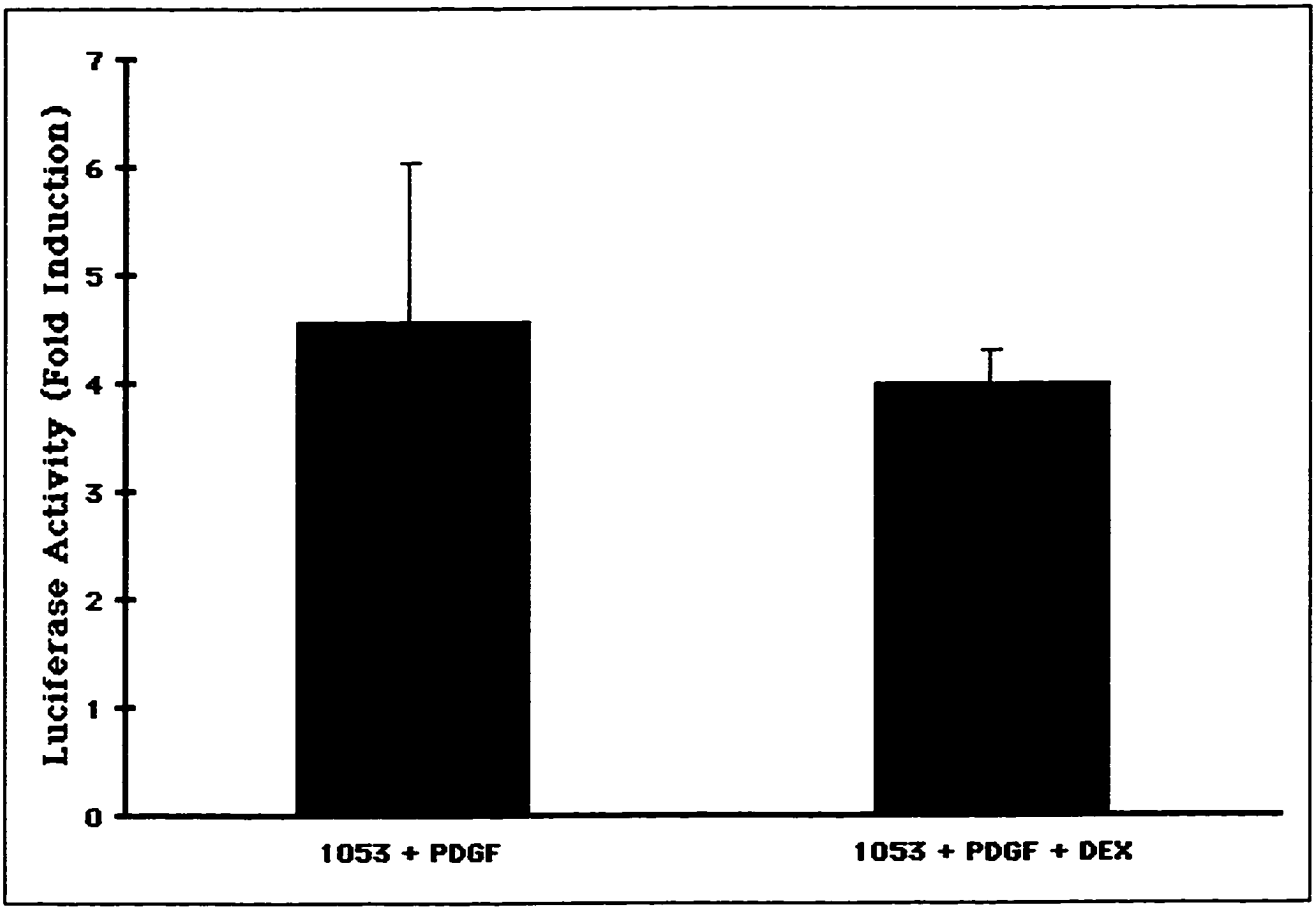


Figure 7. Putative *cis*-acting elements in the proximal rat *MCP-1* promoter

The flag at position +1 indicates the start of transcription. Negative numbers indicate positions of GATA-1 and AP-1 consensus sequences relative to the transcription start site. The promoter sequence was analysed for presence of known *cis*-acting elements using MatInspector software (online access at genomatix.gsf.de).

Figure 7

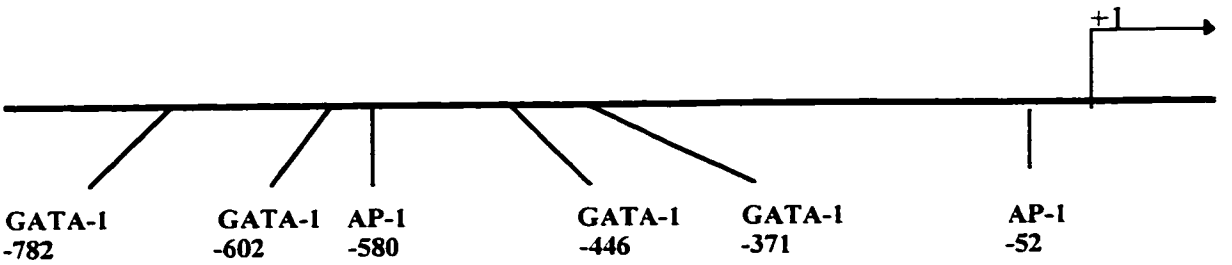


Figure 8. Deletion analysis of the *MCP-1* promoter in VSMC

A) Subconfluent VSMC were transiently transfected with luciferase reporter constructs containing 5' deletions of the *MCP-1* promoter region (numbering is relative to the transcription start site) and stimulated with PDGF BB (20 ng/ml) for 6 hours. Luciferase activity from triplicate experiments is normalized to co-transfected human growth hormone (GH) and expressed as fold induction over levels in non-stimulated transiently transfected VSMC. Error bars are ± SEM. $p<0.01$ between -146 and -128 or -84 and <0.05 between -146 and -104; $p<0.05$ between -84 and -59. **Inset:** agonist specificity of the reporter construct pJE-84. Concentrations are as listed in Figure 2 for PDGF BB (**BB**), PDGF AA (**AA**), a-thrombin (**Thr**), and Ang (**Ang**).

B) A schematic diagram of the constructs pJE-261 through pJE-42. The flag indicates the cap site. Box E1 represents Element 1, box E2 represents Element 2.

Figure 8A

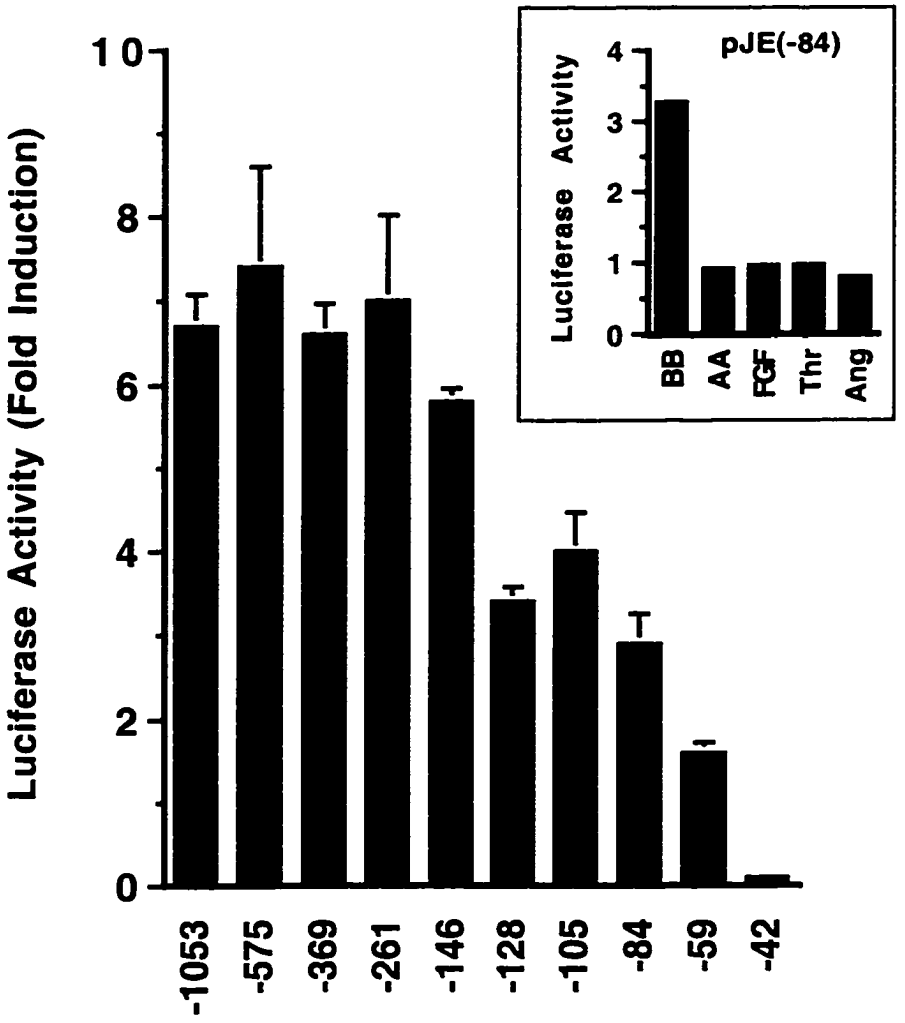


Figure 8B

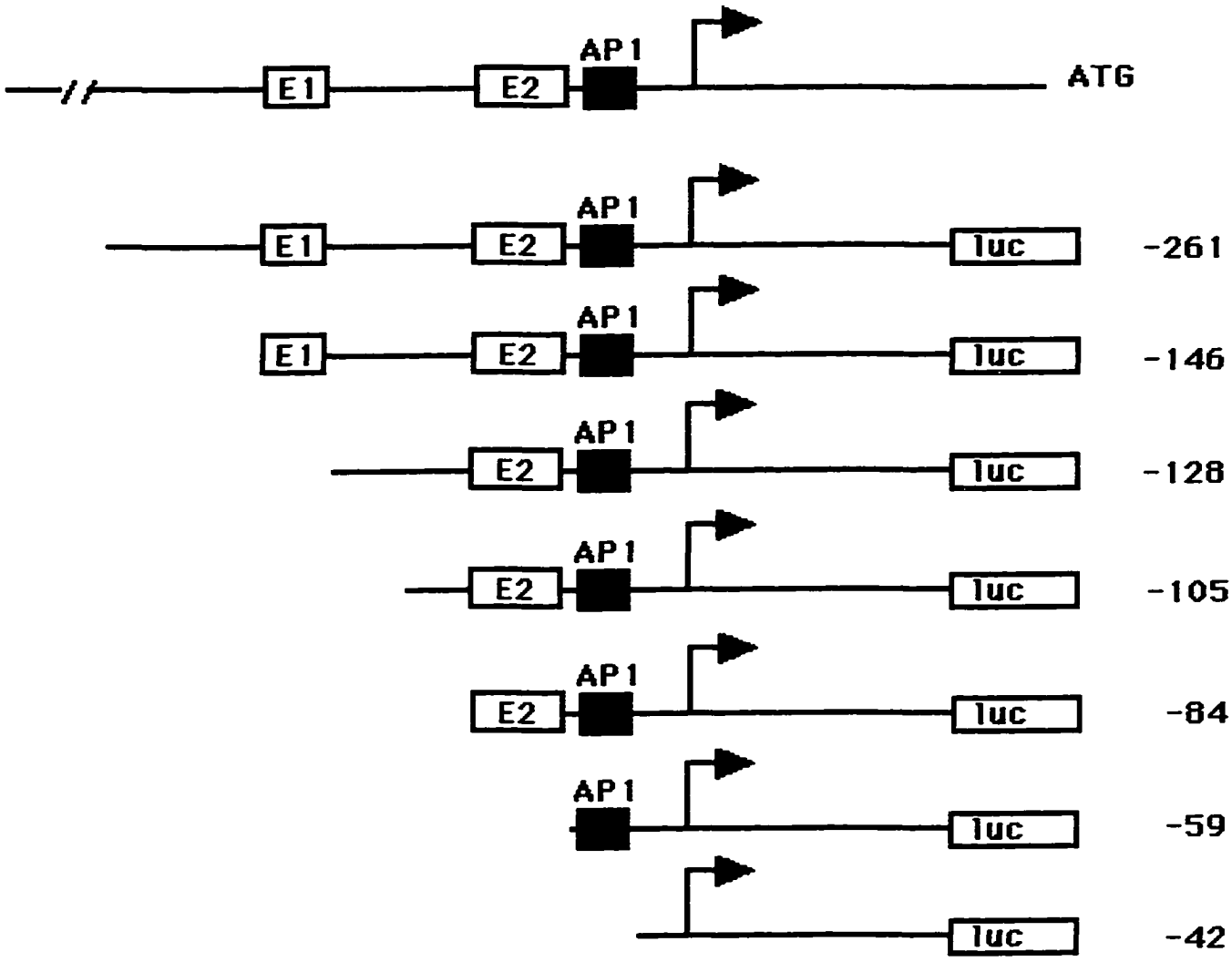


Figure 9. Diagram of genomic regions highly homologous to Elements 1 and 2

In each alignment, the upper strand represents the wild type sequence of Element 1 or Element 2. Identical bases in the corresponding regions are underlined; numbers on the sides of the homologous sequences indicate the position of the region in the genomic sequence. The results were obtained using BLAST search protocol, version 2.0.5 (May 5, 1998).

Figure 9

Element 1: four homologous sequences

1. **H. sapiens gastrin gene, promoter sequence (EPD 25015): 84% homology**

```
5′   TCCAAGGGCTCGGCACTTA   3′
     ||  |||  ||||||||||||
193  TCAGAGGCCTCGGCACTTA   175
```

2. **M. musculus keratin gene, promoter sequence (EPD 32005): 74% homology**

```
5′   TCCAAGGGCTCGGCACTTA   3′
     |  ||  ||||||||||||
463  TATAAAGGCTCGGCACTGT   481
```

3. **M. musculus cathepsin B gene, 5' region (X76621): 61% homology**

```
5′   TCCAAGGGCTCGGCACTTA   3′
     |||||  ||      ||  ||
529  TCCAAAGGAAATACAGTTC   547
```

4. **Aspergillus niger PX18 gene, promoter sequence (M90701): 61% homology**

```
5′   TCCAAGGGCTCGGCACTTA   3′
     |||||||         |||
2278 TCCAAGGGTCACTAGCTTT   2296
```

Element 2: one homologous sequence

M. musculus MIP-2 gene, promoter region (S61346): 48% homology

```
5′   TGATGCTGCTCCTTGGCACCAACCAC 3′
     |||||       ||  | | | ||
291  TGATGACATAGCTCAGTAACCACTGT 316
```

Figure 10. Deletion analysis of the *MCP-1* promoter: role of Element 1

A) Reporter constructs pJE-84(1S) and pJE-84(1AS) contain Element 1 directly upstream of Element 2 in sense and antisense orientations, respectively. pJE-128(1AS) contains Element 1 in its correct location, but in the antisense orientation. pJE-59(1S) contains Element 1, in the sense orientation, directly upstream of the AP-1 site and in the absence of Element 2. The constructs were transiently expressed in VSMC and stimulated with PDGF (20 ng/ml) for 6 hours. Luciferase activity from triplicate experiments performed on two different VSMC strains is normalized to co-transfected human growth hormone (GH) and expressed as fold induction over levels in non-stimulated VSMC. The reporter construct pJE-1053 was used in each transfection assay as a control for the full response to PDGF. The construct pJE-59, containing the AP-1 site, was used as a control for the minimal promoter; the PDGF-induced fold increase in normalized luciferase activity for pJE-59 is represented by the dotted line. Error bars are ± SEM. $p<0.01$ between unstimulated and PDGF-stimulated constructs pJE-84(1S), pJE-84(1AS), and pJE-128(1AS). $p<0.05$ between the same constructs and PDGF-stimulated pJE-59. NS between PDGF-stimulated pJE-59(1S) and pJE-59.

B) A schematic representation of the constructs pJE-84, pJE-84(1S), pJE-84(1AS), pJE-128(1AS), and pJE-59(1S). The flag indicates the cap site. Box E1 represents Element 1, box E2 represents Element 2. Arrows above the boxes indicate the orientation of elements 1 and 2 in each construct..

Figure 10A

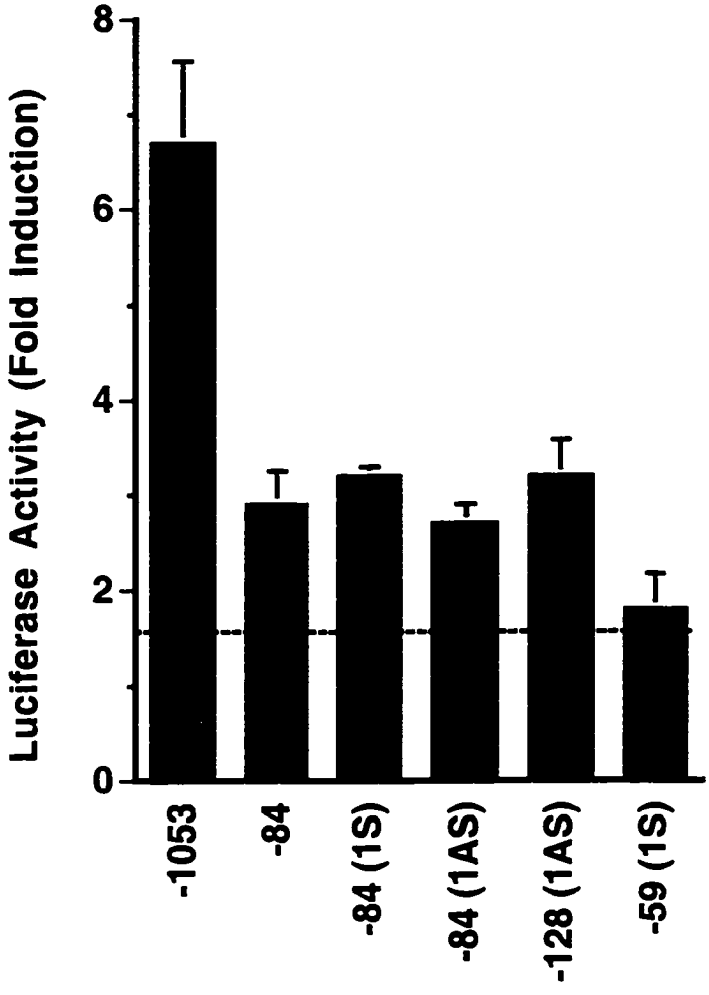


Figure 10B

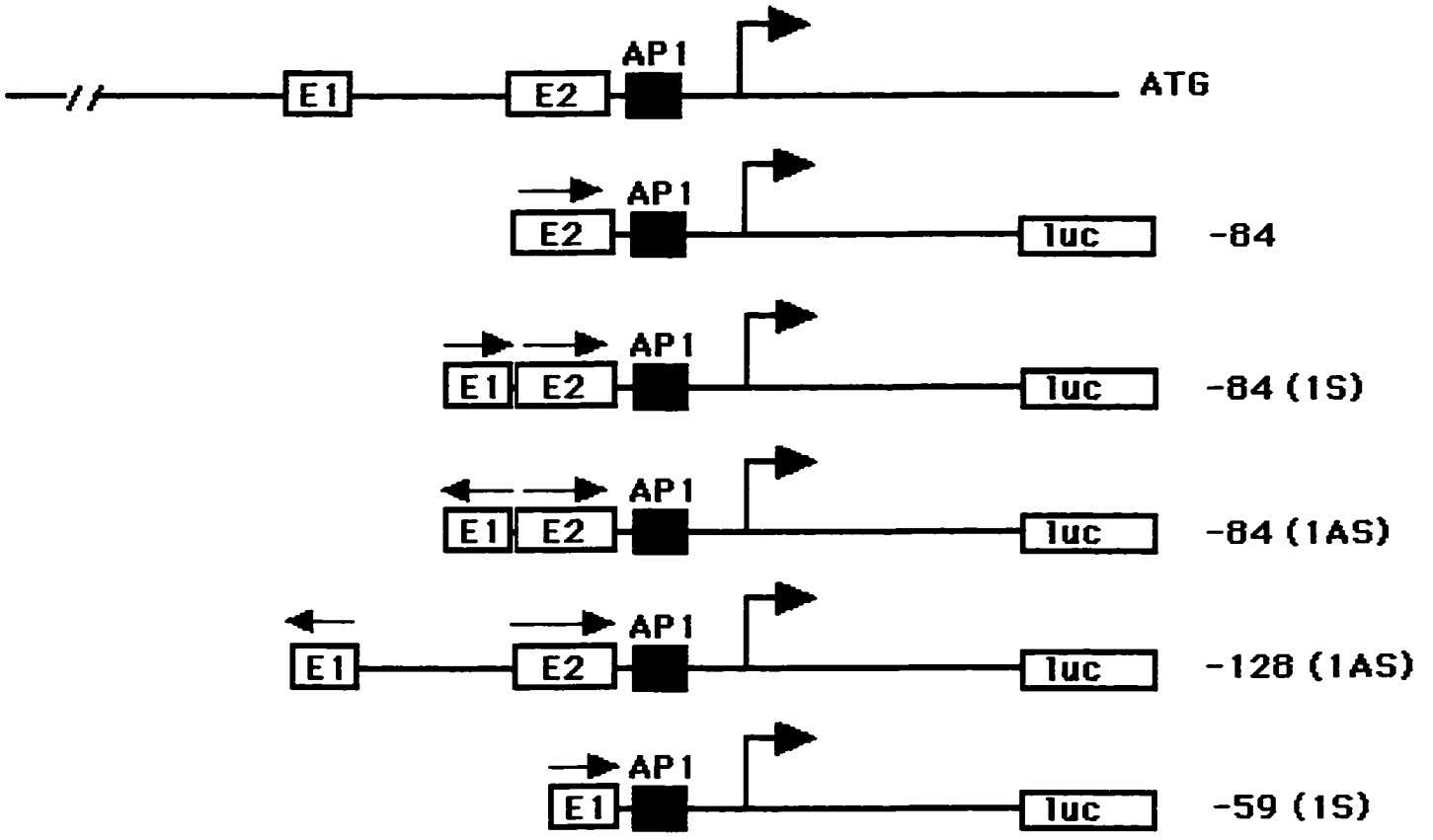


Figure 11. Effects of Elements 1 and 2 on SV-40 promoter

Constructs pGL2(JE S) and pGL2(JE AS) were transiently expressed in VSMC and stimulated with PDGF BB (20 ng/ml) for 6 hours. In these transfection assays, the reporter construct pJE-146 served as a positive control for PDGF induction. Luciferase activity from duplicate experiments is normalized to co-transfected human growth hormone (GH) and expressed as fold induction over levels in non-stimulated transiently transfected VSMC. Error bars are ± SEM.

Figure 11

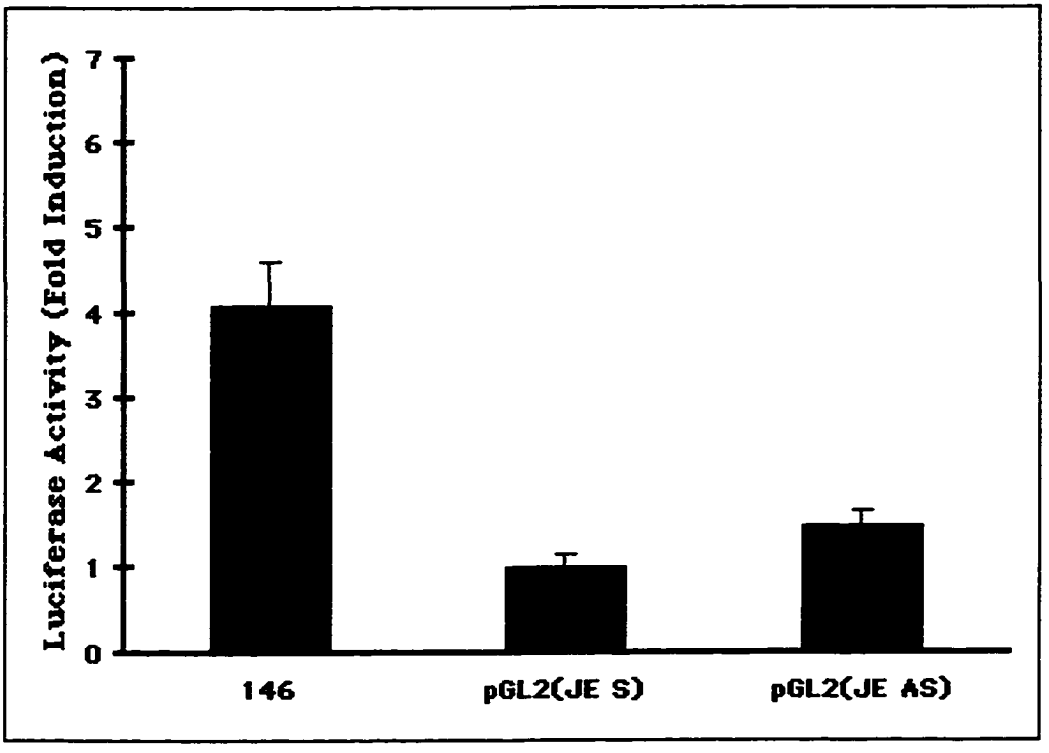
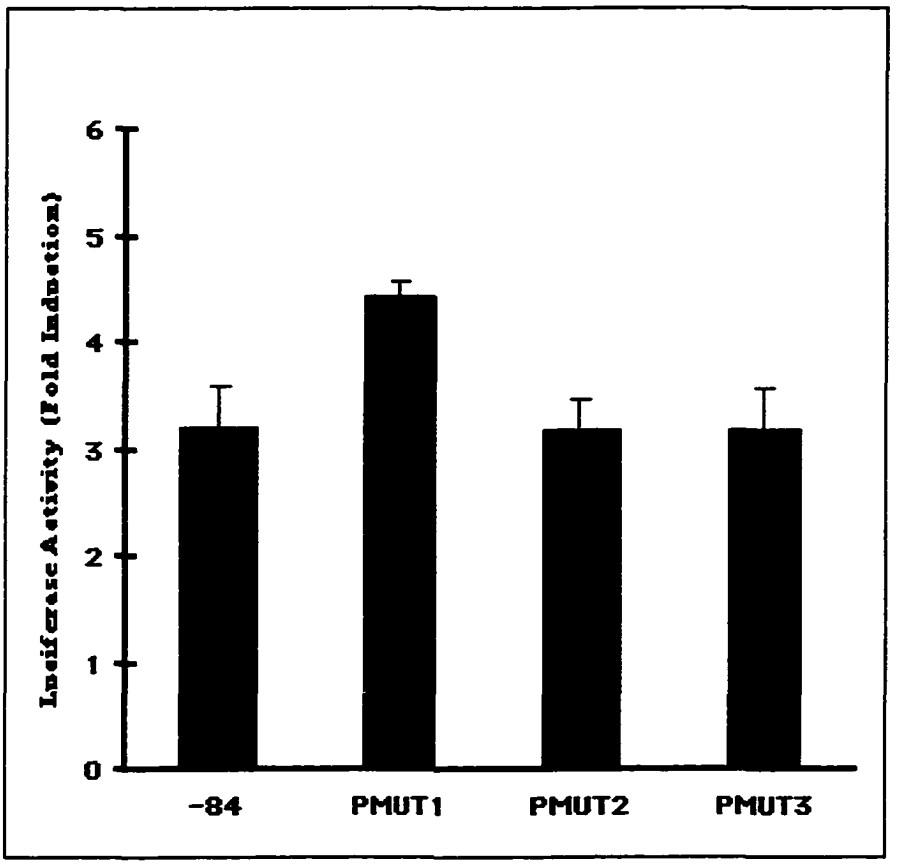


Figure 12. Mutation analysis of Element 2: effect of point mutations

Four luciferase reporter constructs containing a 126 bp *MCP-1* promoter sequence with either a wild type Element 2 sequence (-84 pJE) or point mutations in three different regions of Element 2 (PMUT 1-3, mutated bases underlined) were transiently expressed in VSMC and stimulated with PDGF (20 ng/ml) for 6 hours. Luciferase activity from duplicate experiments is normalized to co-transfected human growth hormone (GH) and expressed as fold induction over levels in non-stimulated VSMC. Error bars are ± SEM.

Figure 12

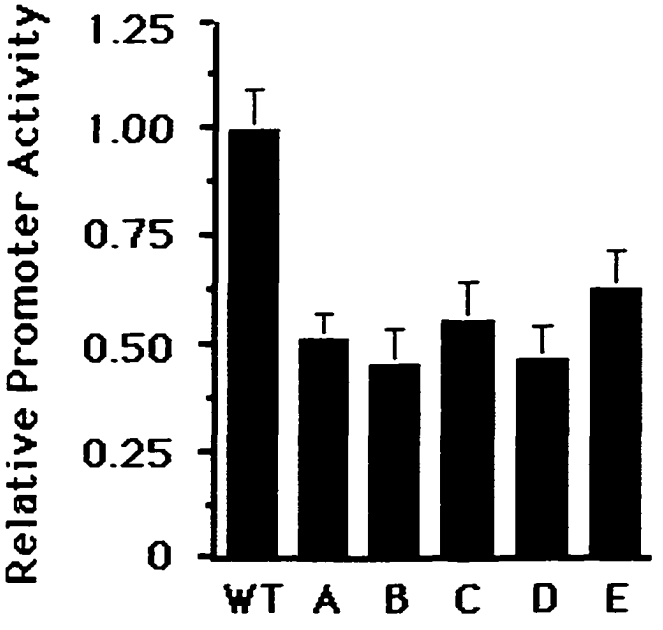


WT	TGATGCTGCTCCTTGGCACCAACCAC
PMUT1	GGATCCTACTCCTTGGCACCAACCAC
PMUT2	TGATGCTGCCCCATGGAACCAACCAC
PMUT3	TGATGCTGCTCCTTGGTACTAGCCAC

Figure 13. Mutation analysis of Element 2: effect of cluster mutations

Six luciferase reporter constructs (WT, A - E) containing a 646 bp *MCP-1* promoter sequence with either a wild type Element 2 (WT), 7-8 bp cluster mutations (sequences underlined) in overlapping regions of Element 2 (A-D), or a mutation of the entire Element 2 (E) were transiently expressed in VSMC isolated from SHR rats and stimulated with PDGF as described in Figure 5. Luciferase activity from triplicate experiments is normalized to co-transfected human growth hormone (GH) and expressed as fold induction over levels in unstimulated VSMC. In the figure, PDGF induction of the wild type construct is presented as 1. Error bars are ± SEM.

Figure 13



WT	TGATGCTGCTCCTTGGCACCAACCAC
A	GACCATGGCTCCTTGGCACCAACCAC
B	TGATGAGTTGAATTGGCACCAACCAC
C	TGATGCTGCTTACCATAGCCAACCAC
D	TGATGCTGCTCCTTGGTGTAGCTAAC
E	GACCAAGTTGAACCATTGTAAGCTTC

Figure 14. Agonist specificity of Element 1

Luciferase reporter constructs pJE-1053 (wild type) and construct "E" harboring a mutation of the entire Element 2 (see Figure 9) were transiently expressed in VSMC isolated from SHR rats and stimulated with PDGF (20 ng/ml) and Ang II (1 μM) for 6 hours. Luciferase activity from duplicate experiments is normalized to co-transfected human growth hormone (GH) and expressed as fold induction over levels in non-stimulated VSMC. Error bars are ± SEM.

Figure 14

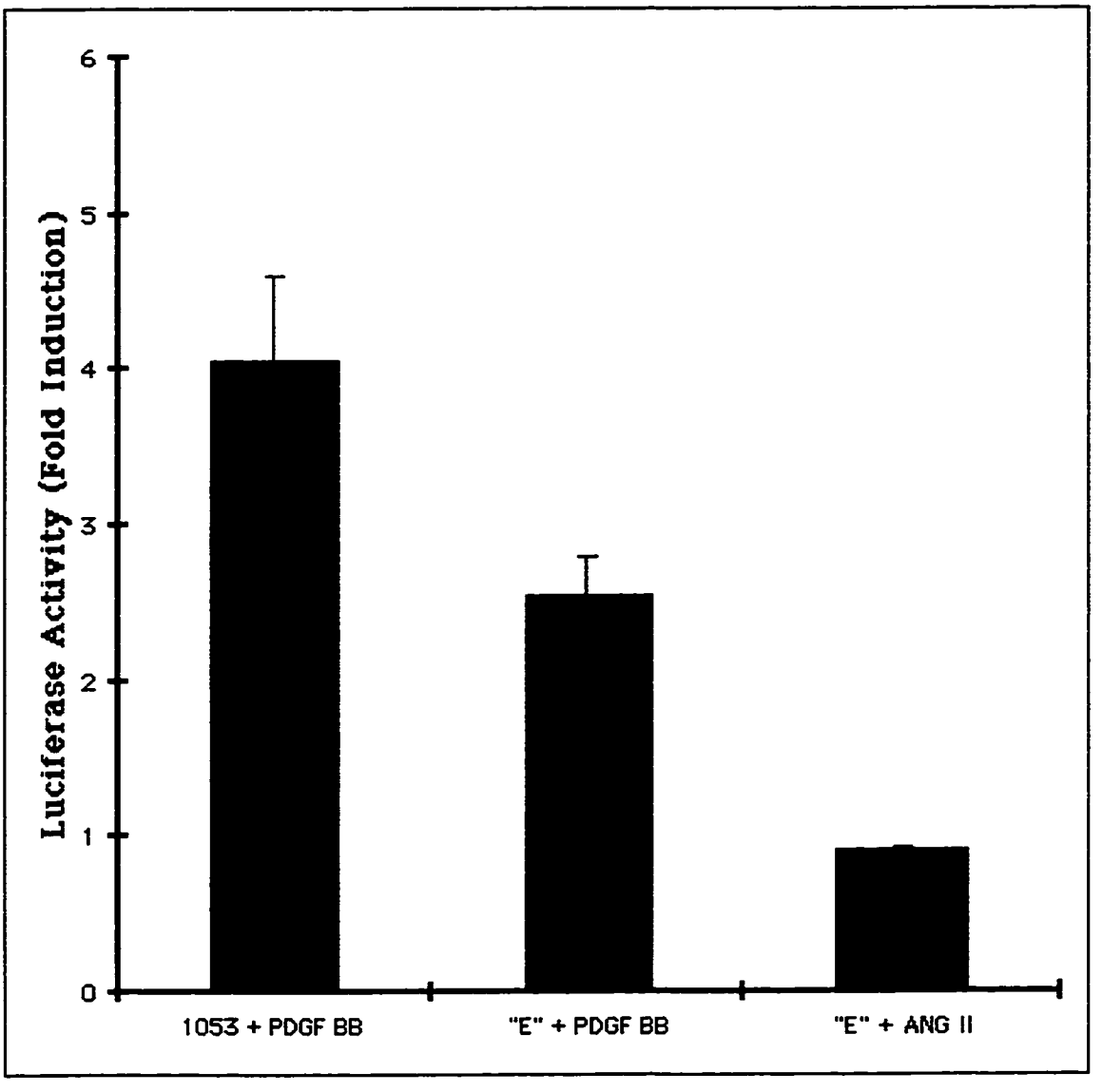


Figure 15. EMSA analysis: band shift pattern observed with the 207 bp probe

EMSA was performed using nuclear extracts from unstimulated VSMC (lane 2) or stimulated with 20 ng/ml PDGF for 15, 30 and 60 minutes, respectively (lanes 3-5). Lane 1 contains the radiolabeled probe (Probe) incubated under identical conditions without nuclear extracts. Lanes 6 and 7 represent a competition in which the radiolabeled probe was incubated with nuclear extracts derived from VSMC stimulated with PDGF for 15 min in the presence of a 300-fold molar excess of unlabeled nonspecific (lane 6) and specific (lane 7) competitor DNA. Arrows represent observed binding complexes.

Figure 15

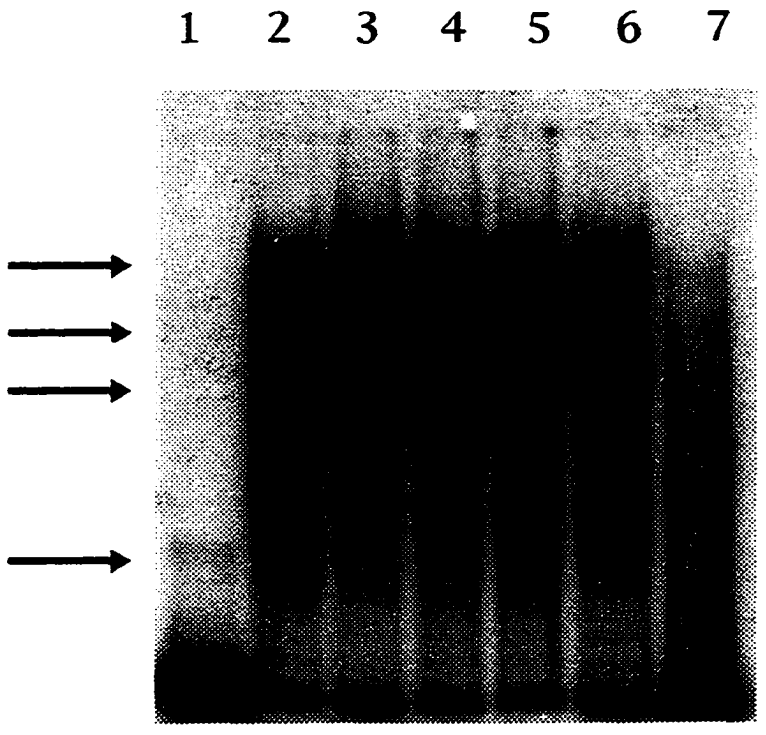


Figure 16. EMSA analysis: band shift pattern observed with probes containing sequences of Element 1 or Element 2.

EMSA was performed using nuclear extracts from unstimulated VSMC (lanes 2 and 8) or stimulated with 20 ng/ml PDGF for 15 min (lanes 3 and 9), 30 min (lanes 4 and 10), and 60 min (lanes 5 and 11). Extracts were incubated with radiolabeled double-stranded oligonucleotides corresponding to Element 1 (lanes 2-6) or Element 2 (lanes 8-12). Lanes 1 and 7 contain the radiolabeled probe incubated under identical conditions without nuclear extracts. Lanes 6 and 12 represent a competition in which the radiolabeled probe was incubated with nuclear extracts derived from VSMC stimulated with PDGF for 15 min in the presence of a 300-fold molar excess of unlabeled specific competitor DNA. Arrows indicate observed binding complexes.

Figure 16

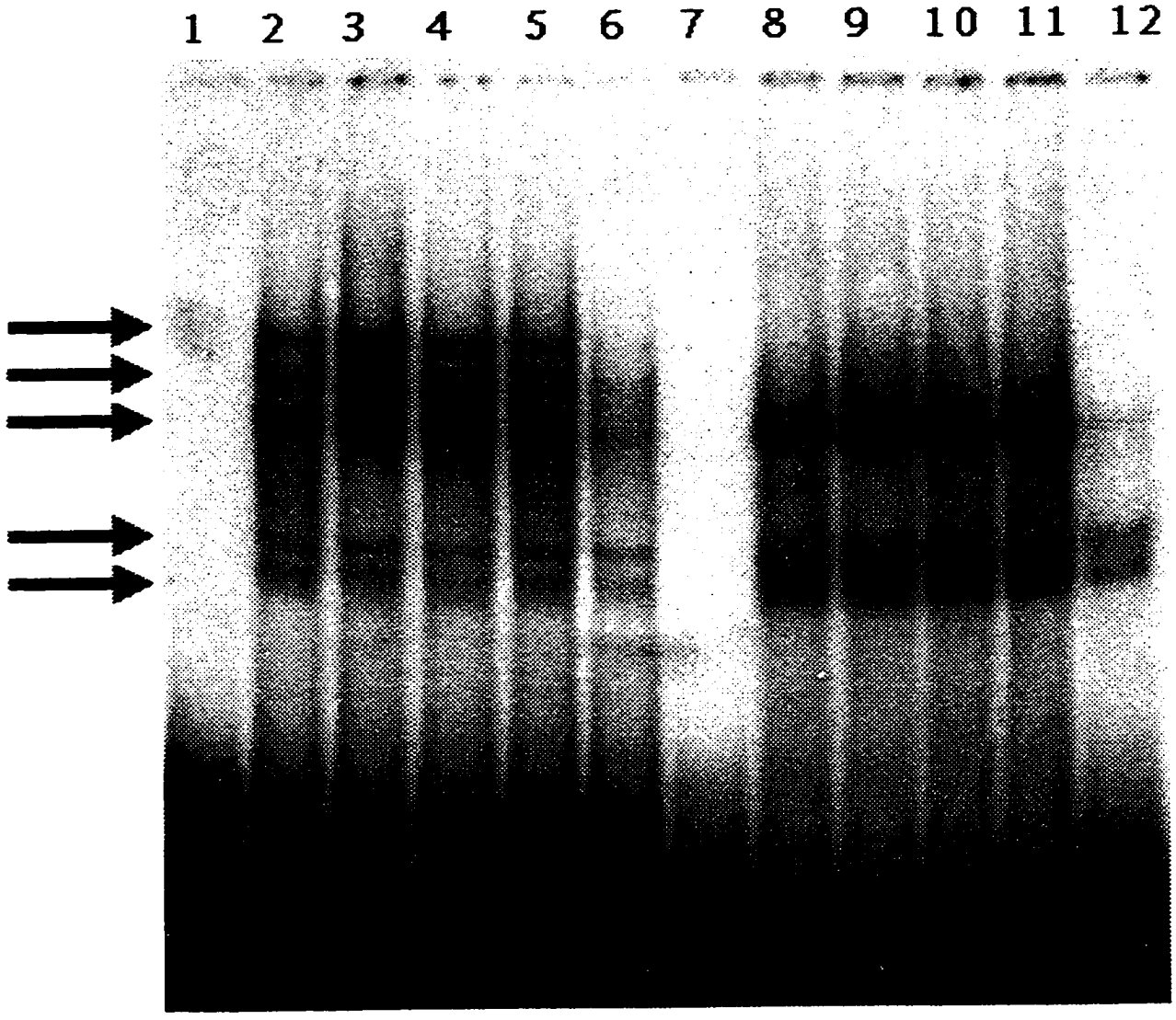


Figure 17. EMSA analysis: band shift pattern observed with probes containing sequences of Element 1 or Element 2 under modified assay conditions

EMSA was performed using nuclear extracts from unstimulated VSMC (0) or stimulated with 20 ng/ml PDGF or 1 μM Ang II for the times indicated. Extracts were incubated with radiolabeled double-stranded oligonucleotides corresponding to Element 1 **(A)** or Element 2 **(B)**. The first lane of each gel contains the radiolabeled probe (Probe) incubated under identical conditions without nuclear extracts. The last lane represents a competition (Comp) in which the radiolabeled probe was incubated with nuclear extracts derived from VSMC stimulated with PDGF for 15 min in the presence of a 300-fold molar excess of unlabeled specific competitor DNA. Arrows represent PDGF-inducible binding complexes. Gels were run under identical conditions.

Figure 17

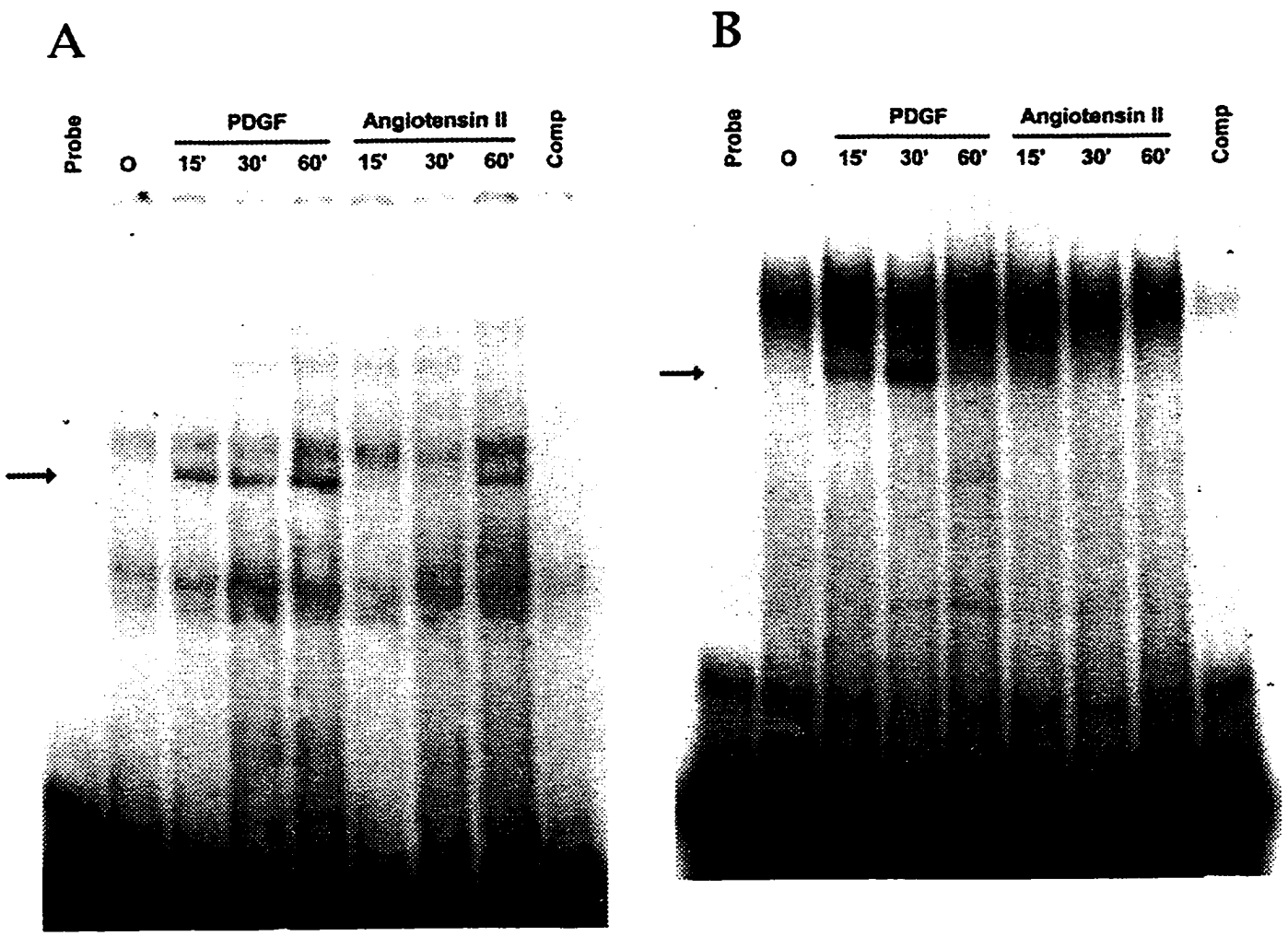


Figure 18. Elements 1 and 2 bind a common regulated protein in EMSA

EMSA was performed as described in Figure 7 with radiolabeled double-stranded oligonucleotides corresponding to Element 1 **(A)** or Element 2 **(B)**. All lanes contain nuclear extracts from VSMC derived from SHR rats and stimulated with PDGF for 30 min. Lanes "E1" and "E2" represent competition experiments in which a 300-fold molar excess of unlabeled Element 1 or Element 2 was used, respectively. Lanes "AP-1 Ab" and "Ets Ab" represent supershift experiments in which supershift antibody reagents were used at a final concentration of 0.25 μg of antibody per μl of incubation mix. Arrows represent PDGF-inducible binding complexes. Gels were run under identical conditions.

Figure 18

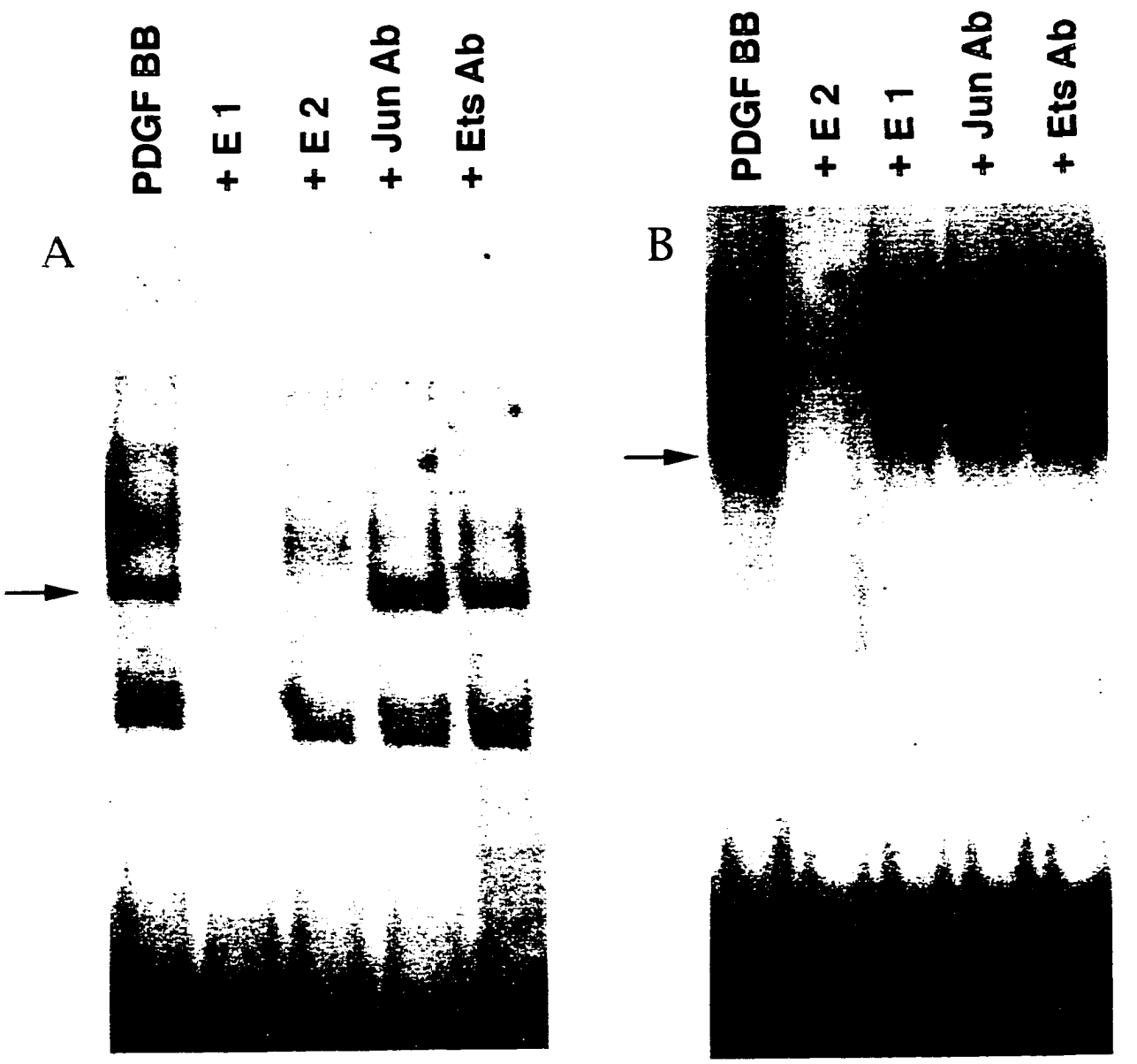


Figure 19. A schematic diagram representing a possible sequence of events leading to upregulation of the rat *MCP-1* promoter by PDGF

Symbols used in the digram are: PDGF R - PDGF receptor: P- protein phosphorylation; E1 - Element 1; E2 - Element 2.

Figure 19

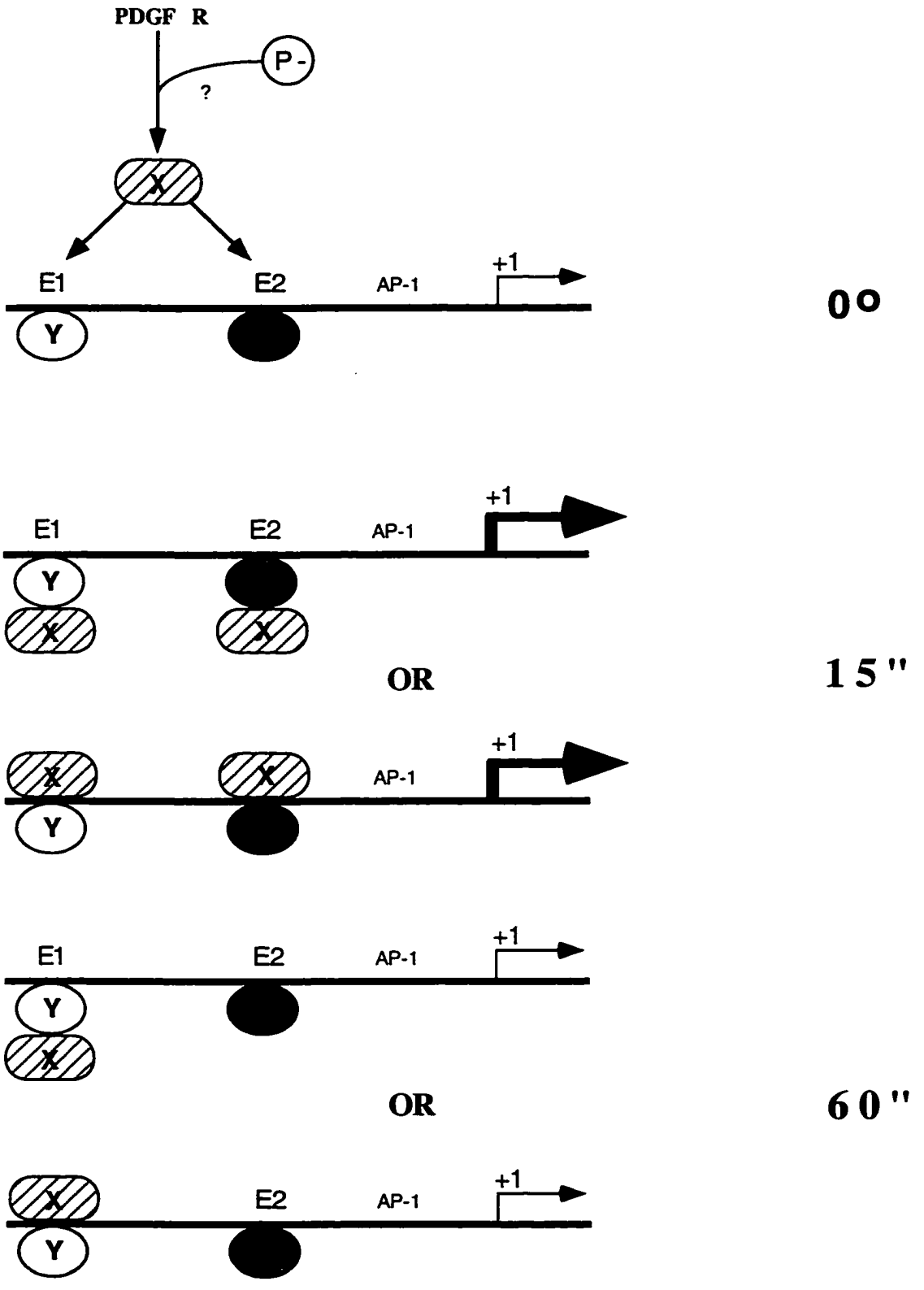


Figure 20. Alignment of the rat, murine and human *MCP-1* promoters

Numbers of bases in each of the three sequences are relative to the transcription start site of the rat, murine or human *MCP-1*.

Box 1: bases corresponding to Element 1; **Box 2:** bases corresponding to Element 2; **Box 3:** bases corresponding to the AP1-like site; **Underlined:** the region in the rat *MCP-1* promoter highly homologous to murine Element 2.

Figure 20

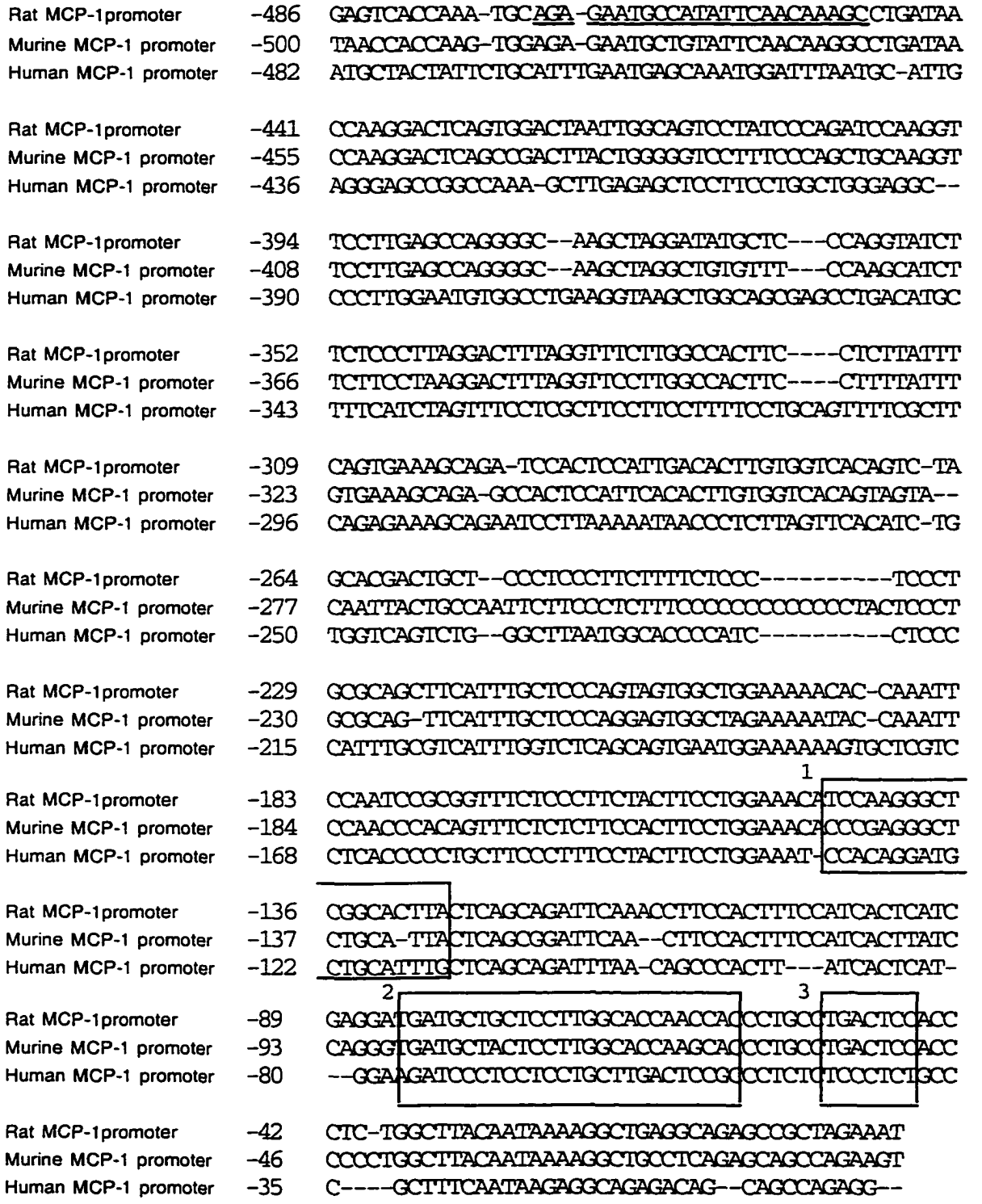
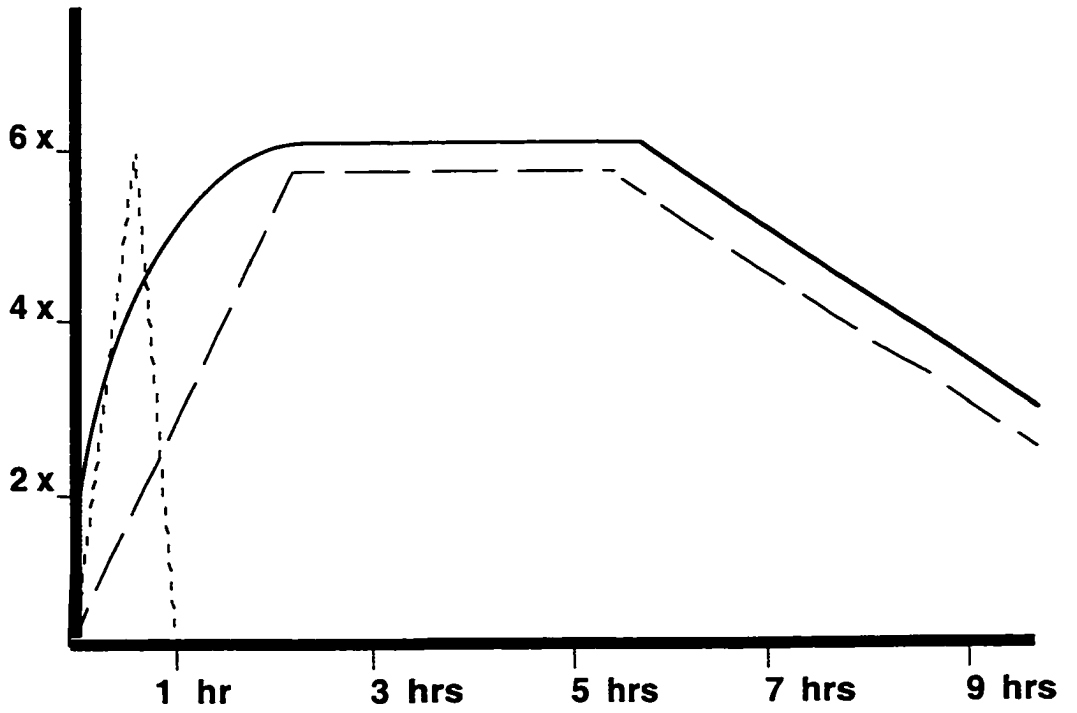


Figure 21. A summary graph representing upregulation of rat *MCP-1* mRNA by PDGF in VSMC

The above diagram is a time course of rat *MCP-1* mRNA upregulation by PDGF in VSMC. The finely interrupted line represents an increase in *MCP-1* transcription. The coarsely interrupted line represents an increase in *MCP-1* mRNA stability. The continuous line is a summary effect of the increases in transcription rate and mRNA stability. The vertical axis is fold induction over levels in non-stimulated VSMC.

Figure21



Chapter 9: Bibliography

Berk BC, Corson MA (1997): Angiotensin II signal transduction in vascular smooth muscle: role of tyrosine kinases. *Circ Res* 80:607-16.

Berk BC, Vekshtein V, Gordon HM, Tsuda T (1989): Angiotensin II-stimulated protein synthesis in cultured vascular smooth muscle cells. *Hypertension* 13:305-14.

Bernstein KE, Berk BC (1993): The biology of angiotensin II receptors. *Am J Kidney Dis* 22:745-54.

Betsholtz C, Johnsson A, Heldin CH, Westermark B (1986): Efficient reversion of simian sarcoma virus-transformation and inhibition of growth factor-induced mitogenesis by suramin. *Proc Natl Acad Sci U S A* 83:6440-4.

Brinson AE, Harding T, Diliberto PA, He Y, Li X, Hunter D, Herman B, Earp HS, Graves LM (1998): Regulation of a calcium-dependent tyrosine kinase in vascular smooth muscle cells by angiotensin II and platelet-derived growth factor. Dependence on calcium and the actin cytoskeleton. *J Biol Chem* 273:1711-8.

Brown Z, Strieter RM, Neild GH, Thompson RC, Kunkel SL, Westwick J (1992): IL-1 receptor antagonist inhibits monocyte chemotactic peptide 1 generation by human mesangial cells. *Kidney Int* 42:95-101.

Charo IF, Myers SJ, Herman A, Franci C, Connolly AJ, Coughlin SR (1994): Molecular cloning and functional expression of two monocyte chemoattractant protein 1 receptors reveals alternative splicing of the carboxyl-terminal tails. *Proc Natl Acad Sci U S A* 91:2752-6.

Current Protocols In Molecular Biology (1997): Vol. 2, Section 12.2, Ausubel FM (ed), John Wiley @ Sons, Inc.

Cushing SD, Berliner JA, Valente AJ, Territo MC, Navab M, Parhami F, Gerrity R, Schwartz CJ, Fogelman AM (1990): Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells. *Proc Natl Acad Sci U S A* 87:5134-8.

Ernofsson M, Siegbahn A (1996): Platelet-derived growth factor-BB and monocyte chemotactic protein-1 induce human peripheral blood monocytes to express tissue factor. *Thromb Res* 83:307-20.

Ernst CA, Zhang YJ, Hancock PR, Rutledge BJ, Corless CL, Rollins BJ (1994): Biochemical and biologic characterization of murine monocyte chemoattractant protein-1. Identification of two functional domains. *J Immunol* 152:3541-9.

Ferns GA, Raines EW, Sprugel KH, Motani AS, Reidy MA, Ross R (1991): Inhibition of neointimal smooth muscle accumulation after angioplasty by an antibody to PDGF. *Science* 253:1129-32.

Freter RR, ALberta AJ, Hwang YG, Wrentmore LA, Stiles DC (1996): PLatelet-derived growth factor induction of the immediate-early gene MCP_1 is mediated by NF-kB AND a 90-KDA PHosphoprotein Coactivator. *J Bioll Chem* 271:17417-17424.

Freter RR, Alberta JA, Lam KK, Stiles CD (1995): A new platelet-derived growth factor-regulated genomic element which binds a serine/threonine phosphoprotein mediates induction of the slow immediate-early gene MCP-1. *Mol Cell Biol* 15:315-25.

Freter RR, Irminger C-J, Porter AJ, Jones DS, Stiles DC (1992): A novel 7-nucleotide motif located in 3' untranslated sequences of the immediate-early gene set mediates platelet-derived growth factor induction of the JE gene. *Mol Cell Biol* 12:5288-5300.

Gabrielsen OS, Huet J (1993): Magnetic DNA affinity purification of yeast transcription factor. *Meth Enzymol* 218: 508-525.

Geisterfer AA, Owens GK (1989): Arginine vasopressin-induced hypertrophy of cultured rat aortic smooth muscle cells. *Hypertension* 14:413-20.

Geisterfer AA, Peach MJ, Owens GK (1988): Angiotensin II induces hypertrophy, not hyperplasia, of cultured rat aortic smooth muscle cells. *Circ Res* 62:749-56.

Gerrity RG (1981): The role of the monocyte in atherogenesis: II. Migration of foam cells from atherosclerotic lesions. *Am J Pathol* 103:191-200.

Gong JH, Clark-Lewis I (1995): Antagonists of monocyte chemoattractant protein 1 identified by modification of functionally critical NH2-terminal residues. *J Exp Med* 181:631-40.

Goppelt-Struebe M, Stroebel M (1995): Synergistic induction of monocyte chemoattractant protein-1 (MCP-1) by platelet-derived growth factor and interleukin-1. *FEBS Lett* 374:375-8.

Grandaliano G, Valente AJ, Abboud HE (1994a): A novel biologic activity of thrombin: stimulation of monocyte chemotactic protein production. *J Exp Med* 179:1737-41.

Grandaliano G, Valente AJ, Rozek MM, Abboud HE (1994b): Gamma interferon stimulates monocyte chemotactic protein (MCP-1) in human mesangial cells. *J Lab Clin Med* 123:282-9.

Green RS, Lieb ME, Weintraub AS, Gacheru SN, Rosenfield CL, Shah S, Kagan HM, Taubman MB (1995): Identification of lysyl oxidase and other platelet-derived growth factor-inducible genes in vascular smooth muscle cells by differential screening. *Lab Invest* 73:476-82.

Gu L, Okada Y, Clinton SK, Gerard C, Sukhova GK, Libby P, Rollins BJ (1998): Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice *Mol Cell* 2:275-81.

Gunn MD, Nelken NA, Liao X, Williams LT (1997): Monocyte chemoattractant protein-1 is sufficient for the chemotaxis of monocytes and lymphocytes in transgenic mice but requires an additional stimulus for inflammatory activation. *Journal of Immunology* 158:376-83.

Hall DJ, Brownlee C, Stiles CD (1989): Interleukin-1 is a potent regulator of JE and KC gene expression in quiescent BALB/c fibroblasts. *J Cell Physiol* 141:154-9.

Hall DJ, Stiles CD (1987): Platelet-derived growth factor-inducible genes respond differentially to at least two distinct intracellular second messengers. *J Biol Chem* 262:15302-8.

Herschman HR (1991): Primary response genes induced by growth factors and tumor promoters. *Annu Rev Biochem* 60:281-319.

Holycross BJ, Peach MJ, Owens GK (1993): Angiotensin II stimulates increased protein synthesis, not increased DNA synthesis, in intact rat aortic segments, in vitro. *J Vasc Res* 30:80-6.

Huang S, Paulauskis JD, Godleski JJ, Kobzik L (1992): Expression of macrophage inflammatory protein-2 and KC mRNA in pulmonary inflammation. *Am J Pathol* 141:981-8.

Introna M, Bast RC, Jr., Tannenbaum CS, Hamilton TA, Adams DO (1987): The effect of LPS on expression of the early "competence" genes JE and KC in murine peritoneal macrophages. *J Immunol* 138:3891-6.

Inui H, Kitami Y, Tani M, Kondo T, Inagami T (1994): Differences in signal transduction between platelet-derived growth factor (PDGF) alpha and beta receptors in vascular smooth muscle cells. PDGF-BB is a potent mitogen, but PDGF-AA promotes only protein synthesis without activation of DNA synthesis [published erratum appears in J Biol Chem 1995 Apr 28;270(17):10358]. *J Biol Chem* 269:30546-52.

Kanner SB, Reynolds AB, Vines RR, Parsons JT (1990): Monoclonal antibodies to individual tyrosine-phosphorylated protein substrates of oncogene-encoded tyrosine kinases. *Proc Natl Acad Sci U S A* 87:3328-32.

Kawahara RS, Deng ZW, Denkinger DJ, Deuel TF (1994): Role of serine/threonine protein kinases in the induction of JE, a platelet-derived growth factor inducible gene. *Biochem Biophys Res Comm* 203:1815-20.

Li S-Y, Kolattukudy EP (1994): Functional role of the cis-acting elements in human monocyte chemotactic protein-1 gene in the regulation of its expression by phorbol ester in human glioblastoma cells. *Mol Cell Biochem* 141:121-128.

Libby P, Schwartz D, Brogi E, Tanaka H, Clinton SK (1992): A cascade model for restenosis. A special case of atherosclerosis progression. *Circulation* 86:III47-52.

Linseman DA, Benjamin CW, Jones DA (1995): Convergence of angiotensin II and platelet-derived growth factor receptor signaling cascades in vascular smooth muscle cells. *J Biol Chem* 270:12563-8.

Loetscher P, Seitz M, Clark-Lewis I, Baggiolini M, Moser B (1994): Monocyte chemotactic proteins MCP-1, MCP-2, and MCP-3 are major attractants for human CD4+ and CD8+ T lymphocytes. *Faseb J* 8:1055-60.

Marmur JD, Poon M, Rossikhina M, Taubman MB (1992): Induction of PDGF-responsive genes in vascular smooth muscle. Implications for the early response to vessel injury. *Circulation* 86:III53-60.

Marra F, Valente AJ, Pinzani M, Abboud HE (1993): Cultured human liver fat-storing cells produce monocyte chemotactic protein-1. Regulation by proinflammatory cytokines. *J Clin Invest* 92:1674-80.

Marumo T, Schini-Kerth VB, Fisslthaler B, Busse R (1997): Platelet-derived growth factor-stimulated superoxide anion production modulates activation of transcription factor NF-kappaB and expression of monocyte chemoattractant protein 1 in human aortic smooth muscle cells. *Circulation* 96:2361-7.

Montmayeur JP, Valius M, Vandenheede J, Kazlauskas A (1997): The platelet-derived growth factor beta receptor triggers multiple cytoplasmic signaling cascades that arrive at the nucleus as distinguishable inputs. *J Biol Chem* 272:32670-8.

Naito S, Shimizu S, Maeda S, Wang J, Paul R, Fagin JA (1998): Ets-1 is an early response gene activated by ET-1 and PDGF-BB in vascular smooth muscle cells. *Am J Physiol* 274:C472-80.

Nelken NA, Coughlin SR, Gordon D, Wilcox JN (1991): Monocyte chemoattractant protein-1 in human atheromatous plaques. *J Clin Invest* 88:1121-7.

Nibbs RJB, Wylie SM, Pragnell IB, Graham GJ (1997): Cloning and characterization of a novel murine beta chemokine receptor, D6. Comparison to three other related macrophage inflammatory protein- 1alpha receptors, CCR-1, CCR-3, and CCR-5. *J Biol Chem* 272:12495-504.

Nordeen SK (1988): Luciferase reporter gene vectors for analysis of promoters and enhancers. *Biotechniques* 6:454-8.

Okuda M, Kawahara Y, Nakayama I, Hoshijima M, Yokoyama M (1995): Angiotensin II transduces its signal to focal adhesions via angiotensin II type 1 receptors in vascular smooth muscle cells. *FEBS Lett* 368:343-7.

Owens GK (1989): Control of hypertrophic versus hyperplastic growth of vascular smooth muscle cells. *Am J Physiol* 257:H1755-65.

Patton WF, Erdjument-Bromage H, Marks AR, Tempst P, Taubman MB (1995): Components of the protein synthesis and folding machinery are induced in vascular smooth muscle cells by hypertrophic and hyperplastic agents. Identification by comparative protein phenotyping and microsequencing. *J Biol Chem* 270:21404-10.

PJ Parker TP (1996): Cell Signalling. In Tooze J (ed), *Cancer Surveys*, Vol 27. Plainview, NY: Cold Spring Harbor Laboratory Press, p 389.

Polte TR, Naftilan AJ, Hanks SK (1994): Focal adhesion kinase is abundant in developing blood vessels and elevation of its phosphotyrosine content in vascular smooth muscle cells is a rapid response to angiotensin II. *J Cell Biochem* 55:106-19.

Poon M, Cohen J, Siddiqui MZ, Fallon JT, Taubman MB (1998): Trapidil inhibits monocyte chemoattractant protein-1 (MCP-1) and macrophage accumulation after balloon arterial injury in rabbits. *Ather Thr Vasc Biol* (submitted).

Poon M, Hsu WC, Bogdanov VY, Taubman MB (1996): Secretion of monocyte chemotactic activity by cultured rat aortic smooth muscle cells in response to PDGF is due predominantly to the induction of JE/MCP-1. *Am J Pathol* 149:307-17.

Poon M, Megyesi J, Green RS, Zhang H, Rollins BJ, Safirstein R, Taubman MB (1991): In vivo and in vitro inhibition of JE gene expression by glucocorticoids. *J Biol Chemistry* 266:22375-9.

Rogers C, Edelman ER, Simon DI (1998): A mAb to the beta2-leukocyte integrin Mac-1 (CD11b/CD18) reduces intimal thickening after angioplasty or stent implantation in rabbits. *Proc Natl Acad Sci U S A* 95:10134-9.

Rollins BJ (1991): JE/MCP-1: an early-response gene encodes a monocyte-specific cytokine. *Cancer Cells* 3:517-24.

Rollins BJ, Morrison ED, Stiles CD (1988): Cloning and expression of JE, a gene inducible by platelet-derived growth factor and whose product has cytokine-like properties. *Proc Nat Acad Sci USA* 85:3738-42.

Rollins BJ, Stier P, Ernst T, Wong GG (1989): The human homolog of the JE gene encodes a monocyte secretory protein. *Mol Cell Biol* 9:4687-95.

Rollins BJ, Yoshimura T, Leonard EJ, Pober JS (1990): Cytokine-activated human endothelial cells synthesize and secrete a monocyte chemoattractant, MCP-1/JE. *Am J Pathol* 136:1229-33.

Ross R (1993): The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* 362:801-9.

Ross R, Raines EW, Bowen-Pope DF (1986): The biology of platelet-derived growth factor. *Cell* 46:155-69.

Rothman A, Wolner B, Button D, Taylor P (1994): Immediate-early gene expression in response to hypertrophic and proliferative stimuli in pulmonary arterial smooth muscle cells. *J Biol Chem* 269:6399-404.

Rovin BH, Yoshiumura T, Tan L (1992): Cytokine-induced production of monocyte chemoattractant protein-1 by cultured human mesangial cells. *J Immunol* 148:2148-53.

Rutherford C, Martin W, Salame M, Carrier M, Anggard E, Ferns G (1997): Substantial inhibition of neo-intimal response to balloon injury in the rat carotid artery using a combination of antibodies to platelet- derived growth factor-BB and basic fibroblast growth factor. *Atherosclerosis* 130:45-51.

Schecter AD, Rollins BJ, Zhang YJ, Charo IF, Fallon JT, Rossikhina M, Giesen PL, Nemerson Y, Taubman MB (1997): Tissue factor is induced by monocyte chemoattractant protein-1 in human aortic smooth muscle and THP-1 cells. *J Biol Chem* 272:28568-73.

Schwartz SM, Heimark RL, Majesky MW (1990): Developmental mechanisms underlying pathology of arteries. *Physiol Rev* 70:1177-209.

Seedorf K (1995): Intracellular signaling by growth factors. *Metabolism* 44:24-32.

Shyy YJ, Li YS, Kolattukudy PE (1990): Structure of human monocyte chemotactic protein gene and its regulation by TPA. *Biochem Biophys Res Commun* 169:346-51.

Sirois MG, Simons M, Edelman ER (1997): Antisense oligonucleotide inhibition of PDGFR-beta receptor subunit expression directs suppression of intimal thickening. *Circulation* 95:669-76.

Steitz SA, Hasegawa K, Chiang SL, Cobb RR, Castro MA, Lobl TJ, Yamada M, Lazarides E, Cardarelli PM (1998): Mapping of MCP-1 functional domains by peptide analysis and site-directed mutagenesis. *FEBS Lett* 430:158-64.

Takeshita A, Chen Y, Watanabe A, Kitano S, Hanazawa S (1995): TGF-beta induces expression of monocyte chemoattractant JE/monocyte chemoattractant protein 1 via transcriptional factor AP-1 induced by protein kinase in osteoblastic cells. *J Immunol* 155:419-26.

Takeya M, Yoshimura T, Leonard EJ, Takahashi K (1993): Detection of monocyte chemoattractant protein-1 in human atherosclerotic lesions by an anti-monocyte chemoattractant protein-1 monoclonal antibody. *Hum Pathol* 24:534-9.

Taubman MB, Berk BC, Izumo S, Tsuda T, Alexander RW, Nadal-Ginard B (1989): Angiotensin II induces c-fos mRNA in aortic smooth muscle. Role of Ca2+ mobilization and protein kinase C activation. *J Biol Chem* 264:526-30.

Taubman MB, Marmur JD, Rosenfield CL, Guha A, Nichtberger S, Nemerson Y (1993): Agonist-mediated tissue factor expression in cultured vascular smooth muscle cells. Role of Ca2+ mobilization and protein kinase C activation. *J Clin Invest* 91:547-52.

Taubman MB, Rollins BJ, Poon M, Marmur J, Green RS, Berk BC, Nadal-Ginard B (1992): JE mRNA accumulates rapidly in aortic injury and in platelet-derived growth factor-stimulated vascular smooth muscle cells. *Circ Res* 70:314-25.

Tekamp-Olson P, Gallegos C, Bauer D, McClain J, Sherry B, Fabre M, van Deventer S, Cerami A (1990): Cloning and characterization of cDNAs for murine macrophage inflammatory protein 2 and its human homologues. *J Exp Med* 172:911-9.

Thiruvikraman SV, Guha A, Roboz J, Taubman MB, Nemerson Y, Fallon JT (1996): In situ localization of tissue factor in human atherosclerotic plaques by binding of digoxigenin-labeled factors VIIa and X [published erratum appears in Lab Invest 1997 Feb;76(2):297-9]. *Lab Invest* 75:451-61.

Timmers MTH, Pronk JG, Bos LJ, Eb dVJA (1990): Analysis of the rat JE gene promoter identifites an AP-1 binding site essential for basal expression but not for TPA induction. *Nucl Acids Res* 18:23-34.

Valente AJ, Rozek MM, Schwartz CJ, Graves DT (1991): Characterization of monocyte chemotactic protein-1 binding to human monocytes. *Biochem Biophys Res Comm* 176:309-14.

van Bilsen M (1997): Signal transduction revisited: recent developments in angiotensin II signaling in the cardiovascular system. *Cardiovasc Res* 36:310-22.

Van Lint J, Ni Y, Valius M, Merlevede W, Vandenheede JR (1998): Platelet-derived growth factor stimulates protein kinase D through the activation of phospholipase Cgamma and protein kinase C. *J Biol Chem* 273:7038-43.

Vignais ML, Sadowski HB, Watling D, Rogers NC, Gilman M (1996): Platelet-derived growth factor induces phosphorylation of multiple JAK family kinases and STAT proteins. *Mol Cell Biol* 16:1759-69.

Waterfield MD, Scrace GT, Whittle N, et al (1983): Platelet-derived growth factor is structurally related to the putative transforming protein p28sis of simian sarcoma virus. *Nature* 304:35-9.

Wilcox JN (1992): Analysis of local gene expression in human atherosclerotic plaques. *J Vasc Surg* 15:913-6.

Williams LT (1989): Signal transduction by the platelet-derived growth factor receptor. *Science* 243:1564-70.

Yla-Herttuala S, Lipton BA, Rosenfeld ME, et al (1991): Expression of monocyte chemoattractant protein 1 in macrophage-rich areas of human and rabbit atherosclerotic lesions. *Proc Natl Acad Sci U S A* 88:5252-6.

Yoshimura T, Robinson EA, Tanaka S, Appella E, Leonard EJ (1989): Purification and amino acid analysis of two human monocyte chemoattractants produced by phytohemagglutinin-stimulated human blood mononuclear leukocytes. *J Immunol* 142:1956-62.

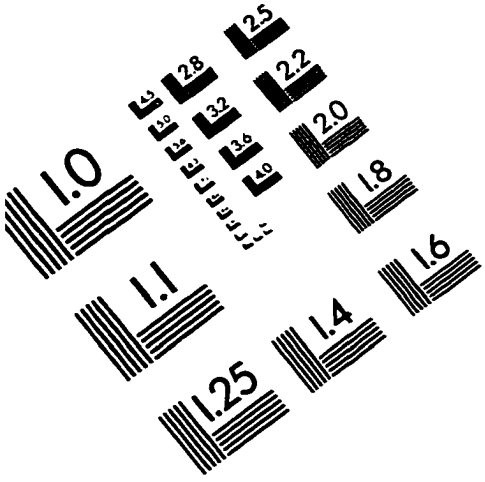
Yu SF, Koerner TJ, Adams DO (1990): Gene regulation in macrophage activation: differential regulation of genes encoding for tumor necrosis factor, interleukin-1, JE, and KC by interferon-gamma and lipopolysaccharide. *J Leukoc Biol* 48:412-9.

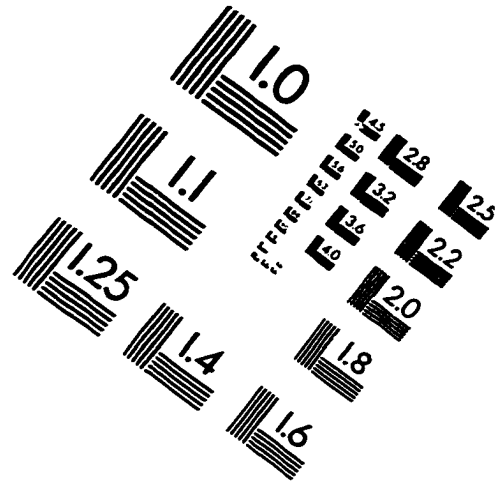
Yu X, Dluz S, Graves DT, et al (1992): Elevated expression of monocyte chemoattractant protein 1 by vascular smooth muscle cells in hypercholesterolemic primates. *Proc Natl Acad Sci U S A* 89:6953-7.

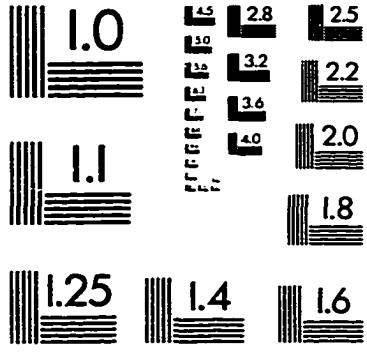
Zhang Y, Rollins BJ (1995): A dominant negative inhibitor indicates that monocyte chemoattractant protein 1 functions as a dimer. *Mol Cell Biol* 15:4851-5.

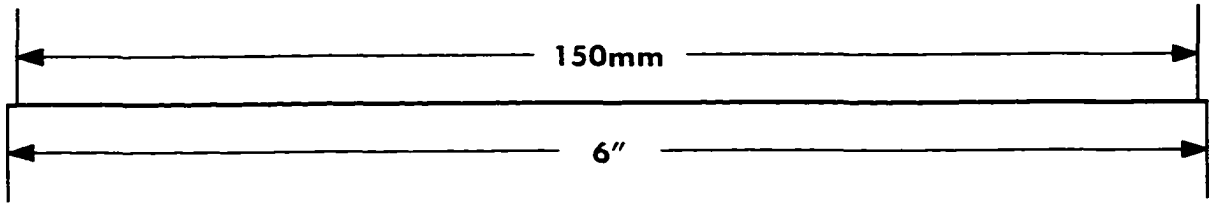
Zhang YJ, Rutledge BJ, Rollins BJ (1994): Structure/activity analysis of human monocyte chemoattractant protein-1 (MCP-1) by mutagenesis. Identification of a mutated protein that inhibits MCP-1-mediated monocyte chemotaxis. *J Biol Chem* 269:15918-24.

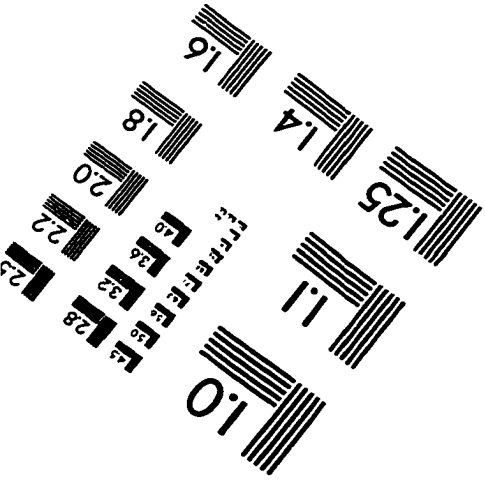
IMAGE EVALUATION TEST TARGET (QA-3)











APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989