MOLECULAR CHARACTERIZATION OF THE PROXIMAL HUMAN ENDOTHELIAL NITRIC OXIDE SYNTHASE (eNOS) PROMOTER

by

Fotula Karantzoulis-Fegaras

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Laboratory Medicine and Pathobiology
University of Toronto

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ABSTRACT

Understanding transcription initiation of the endothelial nitric oxide synthase (eNOS) gene appears pivotal to gaining a comprehensive view of NO biology in the blood vessel wall. The present thesis focused upon a detailed dissection of the functionally important cis-DNA elements and the multiprotein complexes implicated in the cooperative control of the constitutive expression of the human eNOS gene in vascular endothelium. Three tightly clustered cis-regulatory regions were identified in the proximal enhancer of the TATA-less eNOS promoter using deletion analysis and linker-scanning mutagenesis: positive regulatory domain I (PRD I) (-104/-95 relative to transcription initiation), PRD II (-144/-115) and PRD III (-44/-25). The nucleoprotein complexes that form upon these regions in endothelial cells contained Ets family members, Sp1 and variants of Sp3, MAZ and YY1. Functional domain studies in Drosophila Schneider cells and endothelial cells revealed examples of positive and negative protein-protein cooperativity involving Sp1, variants of Sp3, Ets-1, Elf-1 and MAZ. The multiprotein complexes formed on the activator recognition sites within this region of the human eNOS promoter identify the existence of a constitutively active enhanceosome in vascular endothelium. Moreover, in vitro DNase I footprinting analyses and nuclear run-off studies also indicate their important contributions to preinitiation complex formation. Surprisingly, episomal expression
studies performed in endothelial and non-endothelial cell types revealed that eNOS promoter/reporter constructs lacked cell-specificity, suggesting that there exist cell-specific mechanisms that repress native eNOS gene expression in non-expressing cell types. We posited that DNA methylation played a role in this cell-specific expression. Sodium bisulfite genomic sequencing revealed a different methylation pattern of the eNOS promoter in endothelial versus non-endothelial cells. Transient transfection of in vitro methylated promoter/reporter constructs revealed a possible role for methylation in transcriptional repression. DNA hypomethylation studies performed with 5-azacytidine resulted in increased expression of eNOS mRNA in cell types that do not normally express the eNOS mRNA transcript. The role of epigenetic regulation in cell-specific eNOS gene expression is a novel finding in the vascular endothelium. We have identified novel concepts with respect to protein-DNA interactions, pre-initiation complex formation and chromatin-based epigenetic mechanisms in vascular endothelium.
I would like to extend my profound gratitude to my doctoral thesis advisor, Dr. Philip A. Marsden. I benefited greatly from his helpful and constructive advice, as well as from his direction and intellectual challenges. I appreciate all his consistent support, encouragement and confidence in me over the past four years. I am also very thankful for the support, expertise and guidance of my thesis advisory committee members, without whom this thesis would not have been possible: Dr. Philip A. Marsden, Dr. Neil Berinstein, Dr. Avrum Gotlieb, Dr. Gary Levy and Dr. Marlene Rabinovitch. In addition, I would like to extend my thanks to Dr. James Ellis and Dr. Vikas Sukhatme for their important participation in my doctoral defense.

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CHAPTER I

INTRODUCTION
CHAPTER I. INTRODUCTION

1.1 NITRIC OXIDE

Nitric oxide (NO) is an endogenous molecule that has been implicated in many physiological and pathophysiological processes in varied tissues. Once regarded as a nuisance compound, a contributor to smog, acid rain and an ozone-destroying pollutant, nitric oxide has emerged as an endogenous gas crucial for vasodilation, neurotransmission and immunity (58, 67, 191). In 1980, Furchgott and Zawadski provided evidence for an obligatory requirement for vascular endothelial cells in acetylcholine (Ach)-induced relaxation of rabbit aortic segments preconstricted with norepinephrine (97). The ability of acetylcholine to relax isolated rabbit aorta was dependent on the presence of an intact endothelial monolayer. When the endothelium was removed, the ability of acetylcholine to vasorelax was abrogated, instead producing vasoconstriction, especially at higher concentrations (97). The authors attributed this phenomenon to an endothelium-dependent relaxing factor (EDRF), a substance that is released from endothelial cells upon activation of muscarinic receptors. Later work by many laboratories demonstrated that EDRF was, in fact, nitric oxide. For example, Furchgott and Ignarro independently proposed that EDRF may be NO or a related nitroso compound (98, 137-139). EDRF and NO shared numerous pharmacological and biochemical properties. Palmer and colleagues also confirmed the identity of EDRF as NO by using bioassay, chemiluminescence measurements and gas chromatography-mass spectroscopy (239).
These studies provided evidence for the role of NO in the cardiovascular system. Nitric oxide is involved in the regulation of vascular tone, blood pressure, body fluid homeostasis and platelet aggregation and adhesion (201, 327). The discovery of NO as a signaling molecule, especially in the cardiovascular system, was recognized for its significance with the awarding of the 1998 Nobel Prize in Physiology or Medicine to Drs. Furchgott, Ignarro and Murad. Impaired production of NO may contribute to important disease processes which are characterized by changes in vascular reactivity, including hypertension (10, 68, 120, 189, 262, 289), atherosclerosis (120, 122, 189, 255, 338, 348) and septic shock (38, 163, 189, 197), among others (342). Thus, understanding the regulation of nitric oxide synthesis is crucial to the understanding of these diseases.

I.2 RELEVANCE TO PHYSIOLOGIC AND PATHOPHYSIOLOGIC PROCESSES

Vascular endothelial cells are located at the interface between the blood and the vessel wall. The endothelium regulates the control of vascular relaxation and contraction, thrombogenesis and fibrinolysis, and platelet activation and inhibition. Thus, the principal functions of endothelium are the maintenance of anticoagulant properties, the physiological control of lumen diameter and the regulation of vascular permeability. Impaired endothelial dysfunction has been shown to contribute to the pathological consequences associated with acute inflammation, wound healing and cardiovascular disorders such as atherosclerosis (226).

The endothelial cells in blood vessels mediate many physiological and pathophysiological processes by expressing gene products which function as vasodilators (nitric oxide, NO), vasoconstrictors (endothelin-1, ET-1), growth factors (platelet-derived growth factors, PDGF),
growth inhibitors (heparin), adhesion molecules (intercellular adhesion molecule-1, ICAM-1), coagulation factors (tissue factor), and chemoattractants (monocyte chemotactic protein-1, MCP-1). In particular maintenance of vascular endothelial-dependent tone involves the factor nitric oxide, NO.

Nitric oxide is the biological compound responsible for endothelium-derived relaxing factor activity (239). NO is a potent vasodilator and plays a substantial role in blood vessel tone, in platelet adherence and aggregation, in leukocyte adhesion to the endothelium, and in suppression of vascular smooth muscle cell proliferation. Nitric oxide induces vasodilation by stimulating soluble guanylate cyclase to produce cGMP. NO diffuses into the adjacent smooth muscle cell layer and activates soluble guanylate cyclase. This stimulates the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP). Increased cGMP levels leads to smooth muscle cell relaxation and subsequent vasodilation of the blood vessel, via activation of cGMP-dependent protein kinases (187).

Numerous cardiovascular disorders have been associated with altered production of NO, such as hypertension and atherosclerosis. Blood pressure reflects a balance between vasodilatory and vasoconstrictor forces within a blood vessel (342). Perturbations in NO, the major vascular smooth muscle relaxant (97), have been noted in hypertension (262, 342). Homozygous knockout eNOS (-/-) mice demonstrated elevated blood pressure in both conscious and anesthetized states (133, 289). This data confirmed the important role of eNOS as a source of vasodilatory NO. In contrast, transgenic mice overexpressing eNOS appeared hypotensive (235). In addition, the eNOS-overexpressing mice also displayed reduced basal vascular tone and exhibited a reduced vasorelaxant response to NO-mediated vasodilators. Therefore, chronic
eNOS overexpression results in the attenuation rather than enhancement of NO-mediated vasorelaxation (235). Forearm blood flow measurements of young patients with mild essential hypertension, demonstrated attenuated endothelium-dependent vasodilation upon intra-arterial infusions of endothelium-dependent vasodilators (188, 241). Administration of a NOS inhibitor, L-NMMA, to hypertensive patients failed to see a reduction in forearm blood flow, suggesting impaired release of basal NO generation (42). Finally, the 33 CA, intron 13, repeat allele of the endothelial NOS gene was found to be associated with essential hypertension in Japanese subjects (221). The role of eNOS in the pathogenesis of hypertension is still being explored.

Hypercholesterolemic and atherosclerotic blood vessels exhibit reduced NO mediated vasorelaxation (64, 194). The presence of endothelial dysfunction and the loss of NO production are early markers of atherogenesis (270, 271). There is evidence for deficiencies in arginine substrate availability (65), alterations of membrane signaling (31) and enhanced degradation of endothelium-derived NO (213). Recent evidence also demonstrates a loss of eNOS expression by endothelial cells over advanced atherosclerotic lesions (338). Quantitative analysis of a large series of human specimens indicated that eNOS mRNA and protein were decreased in vessels with moderate and severe atherosclerosis compared to normal vessels (338). High LDL cholesterol levels have also been linked to the decrease in basal and stimulated NO production through alteration of eNOS activity, mainly through the promotion of caveolin/eNOS interaction (88). Oxidized LDL has been reported to decrease eNOS mRNA transcript levels in vascular endothelial cells (184). Lysophosphatidylcholine, a component of atherosclerotic lesions, has also been shown to transcriptionally induce eNOS (54, 356). This may initiate a protective response that limits the progression of the atherosclerotic lesion.
It is clear that the understanding of the regulation of eNOS gene expression is vital for potential therapeutic advances in these cardiovascular disorders. A clear understanding of the mechanism of the transcriptional regulation of the basal expression of eNOS is necessary. Thus by studying the mechanisms underlying transcription of the eNOS gene, further mechanistic insight into eNOS gene expression, in settings of disease, may evolve. This will ultimately lead to advances in gene transfer therapies.

1.3 NITRIC OXIDE SYNTHASES

NO is synthesized in mammalian cells by a family of proteins named nitric oxide synthases (NOS). These proteins belong to a class of enzymes that are complex P450-like hemeproteins which catalyze the 5-electron oxidation of the terminal guanido nitrogen of the amino acid L-arginine to form L-citrulline and nitric oxide via the formation of intermediates NG-monomethyl-L-arginine (201). This reaction involves molecular oxygen and NADPH as cosubstrates, as well as a number of other redox cofactors including enzyme-bound heme, reduced thiols, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and (6R)-5.6.7.8-tetrahydrobiopterin (BH4) (177, 212, 327). All three NO synthase isoforms were found to have a bidomain structure consisting of an N-terminal oxygenase and a C-terminal reductase domain. The oxygenase contains prosthetic heme as the reaction center. in addition to BH4 and L-arginine binding sites, while the reductase contains binding sites for NADPH, FAD and FMN (104, 105, 208, 290). Each domain can exist and function independently (299, 321). However, coordinated NO production requires homodimerization of NOS enzyme (5, 13, 51, 300, 314, 347). It has been suggested that NOS dimer interaction involves only the oxygenase domains of either monomer while the reductase domains represent independent monomeric extensions. Recent findings
indicate an alternative quaternary structure for functional endothelial and neuronal NO synthase.

The subunit association of these distinct isoforms involves not only head to head interactions of oxygenase domains but also tail to tail interactions of reductase domains, and head to tail interactions between oxygenase and reductase domains (321).

Nitric oxide synthesis is L-arginine-dependent (126, 143) and is inhibited by structural analogues (substitution of guanidino nitrogen with allyl, ethyl, methyl, succinyl, or nitro groups exhibits inhibitory activity) (125, 135, 143). and addition of excess L-arginine, but not the biologically inactive enantiomer D-arginine, reversed the inhibition in a competitive fashion (125, 136). The first analog described was $\text{NG}^\text{-monomethyl-L-arginine}$ (L-NMMA), which acted as a potent, reversible and stereoselective inhibitor of L-arginine-dependent NO synthesis (125).

There are three human NOS isoforms known to date: neuronal NOS (nNOS, NOS 1), inducible NOS (iNOS, NOS 2), endothelial NOS (eNOS, NOS 3) (327). Their designations nNOS, iNOS and eNOS derive from the fact that they were originally purified from neuronal tissues, immune-activated macrophages and vascular endothelium, respectively (327). For all three NOS enzymes, NO synthesis depends upon the enzyme’s binding of calmodulin, the ubiquitous calcium regulatory protein. Increases in resting intracellular calcium concentration are required for eNOS and nNOS to bind to calmodulin and subsequently become fully activated (358). The binding of the calcium-calmodulin complex results in a conformational change in the NOS protein, thereby allowing electrons to be shuttled from NADPH in the reductase domain to the catalytic heme site in the oxidative domain (1, 358). In contrast, iNOS appears to bind calmodulin with extremely high affinity even at low concentrations of intracellular $\text{Ca}^{2+}$ (50).
The genes encoding the various isoforms of the human NOS proteins are distinct and found on chromosomes 12 (nNOS), 17 (iNOS) and 7 (eNOS) (37, 47, 175, 190, 195, 204, 220, 228, 282, 346). While nNOS and iNOS are widely expressed in an array of different cell types and tissues, the expression of eNOS is relatively restricted to the vascular endothelium. Several lines of evidence indicate that the patterns of expression and mechanisms of regulation of the different isoforms are quite distinct. For instance, the iNOS 5'-flanking regions contain cis-acting DNA elements that are involved in cytokine-mediated gene expression, such as NF-κB, and IRF-1 (47). Multiple promoters regulate the nNOS gene, from which distinct mRNA transcripts are created (327). The regulation of human eNOS gene expression is a newer area of study.

1.4 ENDOTHELIAL NITRIC OXIDE SYNTHASE (eNOS)

Our laboratory reported the isolation and characterization of complementary and genomic clones for eNOS (204)(202). Genomic characterization revealed that the human eNOS gene is composed of 26 exons and 25 introns which are distributed over 21 kilobases of human genomic DNA (203, 214, 219). The transcription start site of this gene was defined by primer extension, S1 nuclease protection, and 5'-RACE analysis (203) (Figure 1A). The gene encodes a messenger RNA of 4052 nucleotides and is present as a single copy in the haploid human genome. The 5' flanking region has been characterized and shown to have no "TATA" consensus sequence. The proximal promoter, however, contains many putative cis-acting DNA elements that are consistent with a constitutively expressed gene found in endothelial cells, such as Sp1 and GATA motifs (340). The 5'-flanking region also contains putative cis-acting DNA elements that are involved in the
Figure 1A. Mapping of the human endothelial NO synthase gene transcriptional start site. 5'-RACE mapping of the 5' end of human endothelial NO synthase mRNA. Total cellular RNA from HUVEC (5 µg) was reverse transcribed with an anti-sense gene-specific primer as previously described (Taken from Marsden, PA. et al J. Biol. Chem. 268: 17478 (1993))
transcriptional response to phorbol esters (AP-1), phorbol esters or cAMP (AP-2), TGF-β (NF-1), cAMP response elements, shear stress and sterols (203, 214, 219) (Figure 1B).

I.5 REGULATION OF eNOS

Endothelial nitric oxide synthase (eNOS) is the enzyme responsible, in major part, for endothelial-derived NO (225, 239, 327). Targeted inactivation of the murine eNOS locus by homologous recombination and physiologic assessment of (-/-) off-spring has reinforced the viewpoint that NO in blood vessels plays a quintessential role in regulation of local blood vessel tonus (133, 289), remodeling of the vascular wall in response to changes in flow or distending hydrostatic pressure and modulation of hemostatic pathways (272). eNOS-deficient mice are viable and fertile, but lack endothelial-dependent vasorelaxation responses (108, 133, 289). Animals are hypertensive at baseline and exhibit exaggerated responses to injuries associated with a hypertensive phenotype, especially in the pulmonary vascular circuit. As our understanding of the contributory roles of NO in the blood vessel wall evolves, so does the need to firmly understand the basic principles governing the regulated expression of the eNOS mRNA transcript and enzyme.

I.5.1 BIOCHEMICAL REGULATION OF eNOS

Biochemical regulation of eNOS at the biochemical and enzymatic level is now better understood. eNOS is a peripheral membrane protein that is localized to specialized cell-surface microdomains known as plasmalemmal caveolae (286). The association of eNOS with the cell membrane is mediated principally by enzyme acylation. N-myristoylation and palmitoylation are necessary
Figure 1B. Potential trans-acting factor binding sites in the 5'-flanking region of the eNOS gene.
for efficient targeting and membrane insertion (40, 41, 268, 269, 286). Plasmalemmal caveola are small membrane invaginations found in most cells which play a functional role as endocytotic carriers and as signal transduction organizing centers (7, 8). Caveolin, a major structural protein of caveola, exists as three distinct isoforms, namely caveolin-1, -2 and -3. eNOS has been shown to directly interact with caveolin-1 in endothelial cells (87, 100, 153) and caveolin-3 in ventricular myocyte cells (19, 87). The interaction between eNOS and caveolin is regulated by Ca\(^{2+}\)-calmodulin (153, 211). The eNOS-caveolin heteromeric complex is disrupted by the addition of calmodulin in a Ca\(^{2+}\)-dependent fashion (153, 211). This proposes a model where eNOS is reciprocally regulated by the inhibitory eNOS-caveolin complex (Figure 2). In turn, this complex is disrupted by the binding of Ca\(^{2+}\)-calmodulin to eNOS, which subsequently leads to eNOS enzymatic activation (153, 211). *In vivo* and *in vitro* protein interaction studies have revealed that the oxygenase domain of eNOS directly interacts with both the N- and C-terminal cytosolic domains of caveolin-1 (153). The role of eNOS palmitoylation in the reversible caveolar targeting of the eNOS-caveolin complex has also been documented (89). Surprisingly, the molecular chaperone Hsp90 is also recruited to eNOS following cellular activation (99). Hsp90 enhances eNOS enzymatic activation via direct protein-protein interaction. Hsp90 may act as an allosteric modulator of eNOS by inducing a conformational change in the enzyme or by stabilizing the dimeric form of eNOS (99). Recent studies have also revealed regulation of eNOS activity in an autoinhibitory fashion. A 52-55 amino acid loop in the reductase domain of the eNOS enzyme functions as an autoinhibitory structural element (227). By extension, this autoinhibitory element also exists in nNOS (227). This insert inhibits activation of eNOS by calmodulin and affects the Ca\(^{2+}\)-dependence of eNOS. The autoinhibitory loop also plays a critical role in governing the net electron transfer ability of the reductase domain by inhibiting electron transfer from the reductase to the heme even when calmodulin is bound (227).
Figure 2. Caveolin-eNOS regulation cycle. (Adapted from Michel, T. J. Clin. Invest. 100: 2146 (1997))
Recent studies have highlighted the important contributions of changes in steady-state eNOS mRNA transcripts to the regulated expression of NO in disease states in vivo and in models of endothelial activation in vitro. For instance, impairment in the bioactivity of endothelial-derived NO may be mediated, in part, through decreased expression of the mRNA and protein for eNOS in atherosclerotic human blood vessels (338). The mechanisms that are operative in the control of eNOS gene expression in development and disease have not been determined. Several lines of evidence indicate that two distinct pathways control eNOS mRNA expression: changes in the rate of transcription and alterations in the stability of the mRNA transcript. Shear stress (228, 257, 315), TGFβ (141), lysophosphatidylcholine (54, 356), hypoxia (11, 209) and protein kinase Cα/ε (183) represent important examples of exogenous stimuli known to modify eNOS gene transcription. Changes in steady state levels of eNOS mRNA have been demonstrated following changes in laminar flow shear stress in vitro and chronic exercise in vivo. It is believed that augmented shear rates of fluid enhance gene transcription both in vitro and in vivo (327).

Consistent with this hypothesis, this laboratory defined a putative shear stress response element in the 5'-flanking region of the human eNOS gene (203). The functional relevance of this finding remains to be determined. eNOS mRNA levels are increased by transforming growth factor β1 in bovine aortic endothelial cells (141). Again, transcription is implicated. A NF-1 site at -1014 with respect to the start site of transcription in the bovine eNOS promoter may mediate this functional response. In human umbilical vein endothelial cells it is now appreciated that lysophosphatidylcholine, a component of atherogenic lipoproteins and atherosclerotic lesions, increases eNOS mRNA levels (356). Nuclear run-off experiments demonstrated that this increase in mRNA was mediated, at least in part, by the transcriptional activation of the eNOS gene via a
phosphatase 2A-dependent increase in Sp1 binding activity (54, 356). Finally, stimulation of either protein kinase Cα or Cε by phorbol esters activates the eNOS promoter in human endothelial cells (183). An intriguing facet of the control of steady-state eNOS mRNA expression in vascular endothelium is the contribution of post-transcriptional regulation (93, 184, 209, 354). The eNOS mRNA transcript normally has a very long half-life in vascular endothelium, usually greater than 24 to 48 h. TNF-α (6, 204, 354), hypoxia (209), entry into the cell-cycle (93), oxidized LDL (184) and Rho GTPase (176) change the metabolic fate of eNOS mRNA transcripts, decreasing the half-life of the mRNA to a few hours. The regulation of eNOS mRNA stability in response to exogenous stimuli, especially the mechanism by which the transcript is degraded, is an evolving story.

Taken together as a whole, it is apparent that the mRNA of this gene is subject to regulation at multiple levels in manners that are plausibly relevant to the pathogenesis of alteration or perturbations of vasomotor control in health and disease, respectively.

1.6  TRANSCRIPTIONAL REGULATION OF GENE EXPRESSION

Each step in the synthesis of a primary mRNA transcript by RNA polymerase II provides a level at which gene expression may be regulated. These steps include activation of gene structure (or chromatin remodeling), preinitiation complex formation, initiation, promoter clearance, transcription elongation and transcription termination. Following these steps, the pre-mRNA is appropriately processed into a functional mature mRNA and translated into a protein, with possible regulation at the level of nuclear RNA processing, mRNA transport, mRNA stability and mRNA translation. Gene expression is frequently controlled at the level of transcription
There are three polymerases that carry out transcription in the eukaryotic cell and these include RNA polymerase I, II and III. RNA polymerase I is localized to the nucleolus and is responsible for transcription of rRNA genes. RNA polymerase II (RNA pol II) is localized to the nucleoplasm and synthesizes heteronuclear RNA, including mRNA from protein-coding genes. Finally, RNA polymerase III, which is localized to the nucleoplasm, is responsible for the synthesis of tRNA, 5S RNA and small nuclear RNA species. Each polymerase interacts with a distinct set of cofactors that facilitate transcription initiation. RNA pol II requires the assistance of a number of transcription factors to recognize a DNA region near the transcription initiation site, known as the promoter. The structure and function of this preinitiation complex will be described below.

### 1.6.1 promoters

Eukaryotic RNA polymerase II (pol II) promoters consist of DNA sequences that include core or basal promoter elements, proximal promoter elements and distal enhancer elements. Core promoter elements include the TATA-box (5' - TATAAA(A) - 3') (24), which is located approximately 25 bp upstream of the transcription start site and the initiator (Inr) element (5' - YYA'INTYY - 3') (146, 296), which tends to be pyrimidine-rich and located at the start site. Core promoters can contain both elements (e.g. IL-6 (TATA (+)/Inr (+)) (260) or adrenomedullin promoter (TATA (+)/Inr (+)) (326)), either element (e.g. the murine T-cell receptor Vbeta 5.2 promoter (TATA (-)/Inr (+)) (48) and the murine p21Cip1/WAF1 promoter (TATA (+)/Inr (-)) (253)) or neither element (e.g. the human eNOS promoter (TATA (-)/Inr (-)) (203)). The function of a promoter is to mediate and control initiation of transcription of a gene located immediately downstream of the promoter (334). The region of the promoter that is sufficient to determine the
precise transcription start site is referred to as the core promoter and usually comprises about 50 nucleotides adjacent to the transcription start site (229, 334). Therefore, a core promoter refers to the structural location at which initiation of transcription occurs. The core promoter can functionally be defined by its capability to assemble the transcription initiation complex and orient it specifically towards the transcription start site of the promoter (334, 355). The concept of the minimal promoter is more of a functional definition referring to the region of DNA that is capable of initiating basal transcription and the region immediately upstream to the minimal promoter constitutes the proximal promoter (extending approximately 300 nucleotides upstream of the start site of transcription) (334). Therefore, the minimal promoter is associated with the minimal functional promoter activity generated by the core promoter and any other functionally important elements contributing to the basal expression of a gene.

1.6.2 TRANSCRIPTION INITIATION IN EUKARYOTES

RNA polymerase II cannot recognize its target promoter directly and initiate transcription in the absence of accessory proteins. RNA pol II relies on the aid of general transcription factors (GTFs), transcriptional activators and cofactors for efficient transcription initiation. The individual GTFs congregate at the core promoter in a highly regulated fashion, forming a complex mass of over 2500 kDa and providing a platform for the recruitment of RNA pol II for efficient transcription initiation to occur (246).

The mechanisms of transcription initiation have not yet been fully worked out. However, substantial advances have been made to elucidate exactly how the RNA polymerase II complexes with the general initiation factors to form a functional transcription unit that is capable of
initiating transcription (Figure 3). Conaway et al nicely summarizes this process (60): The first stage involves the transcription factor TFIID (TBP, the TATA-binding protein, and TAFs, the TBP-associated factors) binding stably to the core promoter of the gene to form the Initial Complex. With the assistance of four other transcription initiation factors, RNA polymerase II subsequently binds to the Initial Complex. The Initial Complex then extends its interactions downstream to form the complete, but inactive, preinitiation complex (PIC) at the start site of transcription. This intermediate is then converted into an activated complex, capable of initiating transcription. The conversion into an activated complex is ATP-dependent. At least five general initiation factors (TFIIA, TFIIIB, TFIID, TFIIE, and TFIIH) have been identified in eukaryotic cells and found to promote selective binding of RNA polymerase II to promoters, as well as support a basal level of transcription (60). TFIIA promotes stable binding of the TFIID to the core promoter (130). TFIIA also interacts with specific transcriptional activators (237). TAF110 (119) and coactivators PC4 (101) and HMG 2 (292). TFIIA is dispensable for accurate initiation but plays an important role in transcriptional activation, functioning as either a coactivator or an anti-repressor (119). TFIIIB is required for selective binding of RNA polymerase II to the Initial Complex. By interacting with the TATA box via TBP and with the polymerase, TFIIIB aligns the polymerase properly on the DNA template. TFIIIB interacts directly with TBP, RNA pol II and other GTFs, including subunits of TFIIF (115). TFIIIB also regulates CTD phosphatase activity by inhibiting the stimulatory activity of TFIIF (45). TFIIE associates stably with the preinitiation complex (109). TFIIH possesses a CTD (carboxyterminal domain) kinase activity (192). TFIIE and TFIIH are both required for promoter clearance or elongation (109). TFIIH also controls RNA polymerase II activity at a postinitiation stage of transcription, by preventing premature arrest by very early elongation complexes just prior to their transition to stably elongating complexes (81). The large subunit
Figure 3. The TFIID complex, a complex containing the TATA-binding protein (TBP) and the TBP-associated factors (TAFs), is the only transcription factor capable of sequence-specific DNA binding. Upon recognition and tight binding of the TATA box, the protein/DNA complex (TFIID/TATA box), preinitiation complex formation begins with the subsequent recruitment of TFIID, to form the TFIID-TFIIA complex. The TFIID-TFIIA complex undergoes a conformational change or isomerization to allow binding of TFIIB and the remaining GTFs. TFIIB, in turn, facilitates the association of a pre-assembled TFIIF-RNA pol IIa (non-phosphorylated form of RNA polymerase II) complex. Finally, preinitiation complex (PIC) assembly is completed with the recruitment of TFIIE, TFIIF and a large aggregate of proteins known as the mediator. The transition from initiation to elongation occurs upon phosphorylation of the carboxy-terminal repeat domain (CTD) of RNA pol IIa to form RNA pol IIo (phosphorylated form). Phosphorylation of CTD allows the removal of TFIIB, TFIIE and TFIIFH promoter clearance by the transcription elongation complex. (Adapted from Osbourne, S. et al Biochem. J. 331: 1 (1998))
carboxy-terminal domain of RNA polymerase II is composed of tandemly repeated copies of a serine-rich heptapeptide with consensus Tyr-Ser-Pro-Thr-Ser-Pro-Ser (62). CTD hyperphosphorylation normally accompanies the transition from initiation to elongation (248). RNA polymerase II is hypophosphorylated when it enters the preinitiation complex and when it pauses shortly after initiation, whereas the actively elongating form of RNA polymerase is hyperphosphorylated (352). The phosphorylation transition of RNA pol II is supported by DRB (5,6-dichloro-1-b-D-ribofuranosylbenzimidazole) treatment, which inhibits the CTD kinase activity of TFIIH and P-TEFb (an elongation factor). This results in an inhibition of chain elongation by RNA pol II (113, 205, 206, 351).

Transcriptional activators/coactivators are believed to function primarily by facilitating formation of functional preinitiation complexes through interactions with one or more components of the basal machinery (256, 308). For example, the Ets transcription factor PU.1 has been shown to interact with TBP in vitro (116). TFIIIB has also been proposed as a target of transcriptional activator proteins. At the adenovirus E4 promoter, TFIIIB assembly is a limiting event that can be facilitated by an activator protein (186). Finally, Sp1 has also been shown to interact with TAF110 (128) and ERM has been shown to bind to TAFII60. TBP and TAFII40 (70).

1.6.3 TRANSCRIPTION ELONGATION IN EUKARYOTES

In addition to the general initiation factors, five general elongation factors (P-TEFb, SII (TFIIS), TFIIF, ELL and Elongin (SIII)) have been identified and found to increase the efficiency of elongation by RNA polymerase II (291, 316). P-TEFb catalyzes the conversion of early elongation complexes, that are prone to terminate, into productive elongation complexes (206).
TFIIS enables the transcription complex to overcome intrinsic sites of pausing and premature termination, as well as providing the ability to read through blocks to elongation caused by bound protein complexes (23, 263). TFIIF is required for both initiation and elongation stages of transcription and increases RNA chain elongation relatively uniformly (343). TFIIF also exhibits an ATP-dependent DNA helicase activity which may melt DNA at the promoter to initiate transcription and at points downstream to facilitate elongation (297). ELL and Elongin both act to increase the overall rate of elongation by RNA polymerase II by suppressing transient pausing by the polymerase at varied sites along the DNA (301).

Together, these elongation factors associate with the transcription initiation complex to convert the complex into a more processive form that is capable of performing efficient elongation. The cooperative effort of these general elongation factors along with other factors, that may play substantial roles at the level of transcription elongation, determine whether a full-length primary transcript will be made.

However, the initiation process and the elongation process are not necessarily distinct processes. Transcription initiation is greatly enhanced by transcriptional activators. These activators function through specific DNA or RNA elements located in the promoter of a gene and they usually act at the level of transcription initiation. Recent evidence suggests that activators can also enhance transcriptional elongation or processivity (30, 169, 337, 351).

Yankulov et al demonstrated that activated transcription complexes possess higher processivity (than nonactivated transcription) and are capable of reading through pause and termination sites efficiently (350). The authors suggested that activators cooperate with the general transcription
factors to convert the RNA polymerase II-transcription complex into a more competent form that is capable of performing transcription elongation. This phenomenon is an integral part of transcription initiation. Ultimately, there are two types of transcription complexes, nonprocessive and processive. In the nonactivated state, the nonprocessive form predominates and transcription mostly arrests prematurely. Thus, these transcription complexes are mostly found in the 5' part of the transcript, as assessed with run-on transcription assays. In the activated state, the processive form predominates, allowing transcription elongation to proceed without any blocks or attenuation (350). Finally, it is the balance between processive and nonprocessive elongation that determines whether a gene will be transcribed and this balance is determined by promoter elements as well as activators that recognize these elements. For example, in the *Drosophila* hsp 70 gene, the GAGA element is required to establish nonprocessive transcription. This nonprocessive transcription is counteracted by the addition of the heat shock factor (the activator) (350). Another example is in the HIV-1 promoter. Here, the TATA box and the inducer of short transcripts (IST) element are important for generating nonprocessive transcription. Processive transcription complexes are formed when the transactivator Tat is introduced into the system (288).

Krumm et al concluded from their studies that RNA polymerase II pausing is a common phenomenon in eukaryotic transcription and it defines a general rate-limiting step after transcription initiation. Their analyses revealed that enhancers have a modest effect on transcription initiation and on release of transcription complexes out of the pause site, but may function primarily to increase the elongation competence of transcription complexes (169). Thus, pausing of transcription complexes is a common feature of all genes and clearance of this pausing is dependent on the transcription factors involved in transactivation (169).
1.6.4 TRANSCRIPTION FACTORS

Trans-acting proteins. transcription factors that bind to cis-regulatory DNA sequences, control transcription. Binding of these proteins generally stimulates transcription. although some regulatory proteins have the ability to repress transcription. Many transcription factors are modular proteins composed of distinct functional domains including a DNA-binding domain and an activation domain (94, 258). The DNA-binding domain is responsible for the specific protein-DNA interactions between a transcription factor and a promoter (52, 341). The activation domain interacts with other proteins to stimulate transcription of a nearby promoter. Transcription factors are often classified according to the type of DNA-binding domain they contain. For example, structural classes of DNA-binding domains include zinc finger proteins (Sp1) (353), winged-helix-turn-helix proteins (Ets) (78), leucine-zipper proteins (fos/jun) (49), homeodomain proteins (Ubx) (18) and helix-loop-helix (MyoD) (172) proteins. On the other hand, activation domains of transcription factors share little homology. When activation domains are tethered to a promoter, greatly elevated levels of transcription are observed. Activation domains may be acidic (asparagine and glutamine-rich. eg. VP16 of the Herpes virus activator protein), glutamine-rich (eg. Sp1) or proline-rich (eg. NF1), among others. Activation domains are identified by using domain-swapping experiments in which regions of the protein are attached to a DNA-binding domain and then assessed for transcriptional activity from a reporter gene (309). Other portions of the protein may also function in oligomerization, interaction with other proteins, ligand binding or transcriptional repression (341). Chimeric protein and deletion studies have demonstrated that various functional domains can function independently, supporting the notion that transcription factors are modular structures (94).
I.6.4.1 Synergism and Cooperativity

In eukaryotes, gene expression is frequently mediated by multiprotein complexes, which are variably composed of a more limited repertoire of proteins that bind DNA in a sequence-specific manner. This regulatory control of transcription is termed combinatorial control (341). The ability of more than one protein to regulate the expression of a gene provides an efficient way to integrate responses to a variety of stimuli or signals using a limited number of proteins (341). The complex interplay among proteins and promoter cis-DNA sequences leads to the appropriate cooperative and synergistic control of transcription. These multiprotein assemblies have been termed enhanceosomes (305). The term enhanceosome has been used to describe the higher order stereo-specific nucleoprotein complexes that forms upon precisely ordered cis-DNA recognition sites. Complexes represent highly coordinated interactions at the DNA-protein and protein-protein level and are composed of transcriptional activators and architectural proteins, among others (305). One well-characterized example of combinatorial interactions among various proteins and distinct regulatory elements is the enhancer of the human interferon β (IFN-β) gene (79, 305). This virus-inducible enhancer consists of overlapping NF-κB, IRF-1 and ATF-c-jun elements. It is the stereospecific architecture of the resulting enhanceosome complex that results in highly specific activation of transcription. The assembly of this precise arrangement of transcription factor binding sites and proteins requires specific protein-protein and protein-DNA interactions (308). The human E-selectin promoter has also been identified as a useful model for biochemical and functional characterization of transcriptional regulation of inducible mRNAs and "enhanceosome" function in vascular endothelium (59). For E-selectin, the activators ATF-2, the p50 and p65 subunits of NK-κB and the architectural high mobility group protein, HMG I(Y), facilitate the robust formation of a highly specific three-dimensional structure on enhancer regions
of the gene following cytokine treatment of the endothelium (59). The requirement of HMG I (Y) for the NF-κB-dependent activation of the E-selectin promoter, as well as the critical role for ATF family members, were reminiscent of properties of the virus-inducible enhancer of the interferon-β (IFN-β) gene (above) (305). Models of nucleoprotein complex formation are also developing for the human ICAM-1 and VCAM-1 genes (59).

1.7 ENDOTHELIAL CELL-SPECIFIC GENE EXPRESSION

Vascular endothelial cells play essential roles in the function and development of the cardiovascular system. However, due to the lack of lineage-specific markers suitable for molecular and biochemical analyses, very little is known about the molecular mechanisms that regulate endothelial cell differentiation. In contrast to the mechanistic details emerging from studies of inducible gene expression, few constitutively expressed endothelial cell-restricted genes have been exhaustively dissected. One well-studied human gene is preproendothelin-1 (159). Functional studies showed that GATA-2 and AP-1 synergistically activate the preproendothelin-1 promoter. GATA-2, the major GATA-binding protein expressed in endothelial cells (340), also has functional importance in the transcription of other endothelial genes such as human von Willebrand factor, P-selectin, VCAM-1 and ICAM-2 (134, 144, 240). However, given the broad tissue distribution of the GATA-2 (178) it cannot be the sole molecular determinant for the cell-type specific expression of these genes. Studies have also investigated the cis-acting DNA elements and trans-acting factors that regulate the transcription of Flt-1, Flk-1/KDR and Tie-2/Tek. CRE (cAMP response element) and Ets motifs cooperate in activating the Flt-1 promoter (324). At least three regions have been identified within the 5’-flanking sequences of the Flk-1/KDR gene containing putative Sp1, AP-2, NF-κB and E-box elements important for functional
activity in endothelial cells (247). Sp1 and Sp3 have been shown to alter Flk-1 expression (121), as well as TFII-I (344). Recent studies with the murine Tie-2 gene identified negative (region I) and positive regulatory elements (regions U and A), though the trans-factors remain to be examined (84). Another group studying the murine Tie promoter identified a role for Ets factors in Tie promoter activity (140). In each of these examples, detailed studies that seek a comprehensive dissection of the functionally important cis-DNA elements and the multiprotein complexes implicated in the cooperative control of constitutive expression in endothelial cells are slowly emerging.

The promoters of genes expressed predominantly in endothelial cells have also been examined in vivo in transgenic mouse studies. For example, transgenic mice containing 2.2 kb of human von Willebrand Factor (vWF) 5'-flanking region demonstrated expression within endothelial cells of the blood vessels of the brain and within the microvasculature of the heart and skeletal muscle. The authors concluded that vWF transgene expression in the murine model did not mirror the known broad endothelial distribution of the native vWF mRNA transcript (2, 3). In contrast, Schlaeger et al identified an endothelial cell-specific transcriptional enhancer derived from the first intron of the murine Tie2 gene (275). Earlier studies with the murine Tie2 gene revealed that 7.2 kb of 5'-flanking sequence was insufficient to recapitulate the endogenous expression pattern of Tie2 (276). This suggested that additional cis-acting regulatory elements were present outside the 5'-promoter region and were required for the complete endothelial cell-specific expression. Further studies revealed an intronic sequence that was critical for endothelial cell-specificity of Tie2 expression in vivo (275). Recently, Fadel et al demonstrated the importance of an octamer sequence in endothelial expression of the murine TIE2 gene using a transgenic mouse model (85). Site-directed mutagenesis of the consensus octamer element resulted in loss of enhancer activity.
and a significant impairment of endothelial expression of the reporter gene (85). Recent work
done with the vascular endothelial-cadherin promoter revealed specific regulatory regions within
the cadherin promoter responsible for endothelial specific expression of a reporter gene in
transgenic mice (111). The authors demonstrated that 2.5 kb of the murine cadherin gene
promoter region was sufficient to direct appropriate transcription in vivo in the endothelium of
adult mice and during vascular development. Moreover, the overall pattern of expression was
similar to that of endogenous cadherin. Finally, Kappel et al demonstrated that the combination
of 5'-flanking sequences with the sequences from the first intron of the Flk-1 gene, the
expression pattern of the transgenic reporter gene was endothelial cell-specific (155).

Our laboratory has recently developed promoter/reporter insertional transgenic mice lines
containing the native murine eNOS promoter (-5200/+28) directing transcription of nuclear-
localized β-galactosidase. Examination of β-galactosidase in the heart, lung, kidney, liver, spleen
and brain of adult mice demonstrated robust signal in large and medium-sized blood vessels (304).
This in vivo expression profile was uniform across multiple founders and not dependent upon
the site of integration into mouse genomic DNA. In contrast, small arterioles, capillaries and
venules were notably negative. Non-endothelial cells were positive only in the brain: the CA1
region of the hippocampus, Purkinje cells of the cerebellar cortex and outer cortical neuronal
layers of the cerebrum. In summary, the findings of this work demonstrated that murine eNOS
genomic regions spanning -5200/+28, relative to the transcription start site, direct expression of a
reporter construct in a fashion that recapitulates the known expression profile of eNOS mRNA
and protein.
1.8 RATIONALE AND RELEVANCE

Little is known with respect to transcriptional mechanisms regulating expression of endothelial cell-specific genes. Using gene transfer techniques, reporter gene products and exogenous genes can now be expressed in the vascular wall of animal models (218). Critical to the development of gene transfer therapies will be a clear understanding of the basic principles that target endogenous genes to cells of endothelial lineage and the molecular mechanisms by which environmental stimuli implicated in disease (e.g. shear, cytokines, sterol or hypoxia) modulate their expression. Thus, by studying the mechanisms underlying transcription of the eNOS gene further mechanistic insight into endothelial cell-specific gene expression may evolve.

1.8.1 eNOS CELL-SPECIFIC EXPRESSION

In situ cRNA hybridization studies performed in a wide variety of human tissues revealed that eNOS mRNA transcripts are relatively endothelial cell-specific (204, 338). This contrasts with the broad tissue distribution of other known members of the human NOS gene family, namely neuronal NOS and inducible NOS. As is the case with nearly all cell-restricted transcripts, some exceptions to the endothelial-restricted expression of eNOS mRNA transcripts have been noted: syncytiotrophoblast of human placental villi (61), pyramidal cells of the CA1 region of the brain (76) and cardiac myocytes (16, 281, 333), among others (273, 287, 311). In situ hybridization revealed the presence of eNOS message in the syncytiotrophoblast of human placental villi (61). Immunohistochemistry studies with pyramidal cells of the CA1 region of the rat hippocampus also demonstrated eNOS expression (76). mRNA and protein expression has also been reported in cultured NCI-H441 human bronchiolar epithelial cells (287). Tracey et al also reported the
identification of an endothelial-like NOS in LLC-PK₁ kidney epithelial cells (311). Recently, expression of eNOS mRNA and protein has been demonstrated in atrial and ventricular cardiac myocytes (333)(16, 281). Expression of eNOS has also been seen in human blood platelets (273). When compared to other genes expressed in vascular endothelium such as preproendothelin-1, endothelin converting enzyme-1, CD31/PECAM or vonWillebrand factor, the mRNA for eNOS is very endothelial-cell specific. mRNAs known to be even more restricted to the vascular endothelium than eNOS are uncommon, but include the endothelial receptor tyrosine kinases Flk-1/KDR, Flt-1, Tie-1, Tie-2/Tek and the cytokine-inducible adhesion molecule E-selectin (267). In the latter case, the human E-selectin promoter has been identified as a useful model for biochemical and functional characterization of transcriptional regulation of inducible mRNAs in vascular endothelium.

1.8.2 TRANSCRIPTIONAL REGULATION OF eNOS

Our laboratory reported the importance of Sp1 and GATA factors in the regulation of the eNOS gene (203). Others have reported similar findings (302, 329, 359). Deletion and mutational analysis revealed essential roles for Sp1 and GATA elements for basal eNOS transcription (302, 329, 359). Sp1 binding was demonstrated with EMSA and in vitro footprinting analysis (302, 359). However, further studies are necessary to elucidate the mechanism of eNOS transcriptional regulation in vascular endothelial cells.

Although both transcription and mRNA stability are important in the regulated expression of eNOS mRNA transcription, the focus of this study will be upon transcription. Therefore, to
address the mechanism of eNOS gene transcription, a more detailed analysis of the proximal promoter or the 5'-flanking region of the eNOS gene is warranted.

The eNOS gene is constitutively expressed by the vascular endothelium, however the transcriptional mechanisms have not been thoroughly investigated thus far. A comprehensive understanding of the interdependent protein-DNA and protein-protein interactions that reciprocally interact with co-activators and the general transcription machinery on the native eNOS promoter is necessary for developing further insight into perturbations of eNOS expression in the diseased blood vessel wall. Based upon this premise, the present study focused upon a detailed dissection of the functionally important cis-DNA elements and the multiprotein complexes implicated in the cooperative control of constitutive expression of the human eNOS gene.
CHAPTER II

CHARACTERIZATION OF THE HUMAN eNOS PROMOTER

(PART I)

Portions of this chapter have been published as:


CHAPTER II. CHARACTERIZATION OF THE HUMAN eNOS PROMOTER (PART I)

II.1 OBJECTIVE AND AIMS

There exists a limited amount of knowledge with respect to transcriptional mechanisms regulating constitutive expression of endothelial cell-specific genes. A comprehensive understanding of the interdependent protein-DNA and protein-protein interactions that reciprocally interact with co-activators and the general transcription machinery on the native eNOS promoter is necessary for developing further insight into perturbations of eNOS expression in the diseased blood vessel wall. Based upon this premise, the present study focuses upon a detailed dissection of the multiprotein complexes that form upon functionally important cis-DNA elements and are implicated in the cooperative control of constitutive expression of the human eNOS gene in vascular endothelial cells. We posit that by studying the mechanisms underlying transcription of the eNOS gene further mechanistic insight into endothelial cell-specific gene expression may evolve.
II.1.1 OBJECTIVE

The overall objective of these studies is to examine the basic regulatory features implicated in the physiologic and pathophysiologic control of human eNOS gene transcription in vascular endothelial cells.

II.1.2 SPECIFIC AIMS

1. The first specific aim of this chapter is to define the functionally important cis-DNA elements in the human eNOS promoter, using deletion mutant construct analysis and linker-scanning mutagenesis of promoter/reporter constructs. The goal is to define the important cis-regulatory DNA elements responsible for 'basal' promoter activity in endothelial cells.

2. The second specific aim is compare eNOS promoter activity in endothelial versus non-endothelial cell types.

II.2 HYPOTHESIS

The 5'-flanking region of the human endothelial nitric oxide synthase gene contains functionally important cis-DNA elements that are responsible for basal promoter activity in vascular endothelial cells. These cis-DNA elements comprise the necessary DNA sequences or promoter used for efficient transcription initiation by the eNOS gene in vascular endothelial cells.
II.3 MATERIALS AND METHODS

Materials. Cell culture media, balanced salt solutions, antibiotics and trypsin-EDTA were obtained from Gibco BRL (Grand Island, NY). Cell culture plates and Teflon scrapers were from Costar (Cambridge, MA). Restriction enzymes were from New England Biolabs (Beverly, MA) and Boehringer Mannheim (Mannheim, Germany). Cell culture grade bovine gelatin was purchased from Sigma Chemical (St. Louis, MO). CPRG (chlorophenol red-β-D-galactopyranoside) was purchased from Boehringer Mannheim (Mannheim, Germany). Coenzyme A and ATP were purchased from Calbiochem (La Jolla, CA). D-luciferin and luminometer cuvettes were purchased from Analytical Luminescence Laboratory/Pharmingen (Ann Arbor, MI). All luciferase assays were carried out on the MonoLight 2010 luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI). All optical density readings were performed on an Ultrospec Plus UV/Visible Spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). Determination of DNA concentration was performed with a DyNA Quant 200 Fluorometer (Hoefer Pharmacia Biotech Inc., Uppsala, Sweden).

Cell Culture. Bovine aortic endothelial cells (BAEC) were isolated from calf aortas. BAEC cultures were propagated in RPMI 1640 medium with 15% supplemented low-endotoxin, bovine serum (Gibco-BRL), 100 U/ml penicillin G sodium and 100 μg/ml streptomycin sulfate. The cell culture was maintained at 37 °C and 5% CO₂. Primary endothelial cell cultures were grown on 100 mm plates coated with 0.2% bovine gelatin. Individual BAEC clones were isolated by using cloning cylinders and trypsin-EDTA. Homogeneous populations of cells were grown to confluence and serially passaged using trypsin-EDTA digestion. Cell cultures were characterized as previously described (93)(204) and passages 3 to 5 were utilized for experiments. HepG2
(human hepatocellular carcinoma) and Cos7 (SV40 transformed, African green monkey kidney cells) were obtained from ATCC (Rockville, MA) and maintained at 37 °C and 5% CO2. HepG2 cells were propagated in α-MEM with 10% supplemented fetal bovine serum, 100 U/ml penicillin G sodium and 100 μg/ml streptomycin sulfate. Cos7 cells were propagated in D-MEM with 10% supplemented fetal bovine serum, 100 U/ml penicillin G sodium and 100 μg/ml streptomycin sulfate. HUVEC (human umbilical vein endothelial cells) were subcultured and maintained as previously described (209). HeLa (human cervical carcinoma) cells were obtained from ATCC (Rockville, MA) and maintained in Dulbecco's modified Eagles Medium (Gibco BRL, Grand Island, NY), supplemented with 10% fetal bovine serum, 100 U/ml penicillin G sodium and 100 μg/ml streptomycin sulfate. The murine embryonal carcinoma cells (F9) (ATCC) were cultured with Dulbecco's Modified Eagles Medium with high glucose (4.5 g/L) (Gibco BRL, Grand Island, NY), 15% fetal bovine serum, 100 U/ml penicillin G sodium and 100 μg/ml streptomycin sulfate. Chinese hamster ovary cells (CHO), obtained from ATCC, were maintained in HAM's F-12 media, supplemented with 10% fetal bovine serum, 100 U/ml penicillin G sodium and 100 μg/ml streptomycin sulfate. Human choriocarcinoma cells (JEG-3) (ATCC) were cultured in Minimum Essential medium (Eagles) (Gibco BRL), supplemented with 10% bovine calf serum, 100 U/ml penicillin G sodium and 100 μg/ml streptomycin sulfate. TGW cells (a human neuroblastoma cell line) were a generous gift of Dr. Toshio Kuroki (Department of Cancer Cell Research, Institute of Medical Sciences, University of Tokyo, Japan). These cells were propagated in α-MEM, supplemented with 10% fetal bovine serum, 100 U/ml penicillin G sodium and 100 μg/ml streptomycin sulfate. SL2 (Drosophila Schneider's line 2) was propagated in Schneider's Drosophila Medium, supplemented with 10% fetal bovine serum, 100 U/ml penicillin G sodium and 100 μg/ml streptomycin sulfate.
Plasmid Construction. -1193/+109 linker mutants. Twelve linker-scanning mutations were created that span a 120 base pair region of the human eNOS promoter from -164 to -45, relative to the start site of transcription (Figure 4). These mutations were incorporated into the pGL2 -1193/+109 construct. The pGL2 -1193/+109 construct was created as previously described (9).

Two types of amplicons, namely an A amplicon (the 5' fragment) and a B amplicon (the 3' fragment) were created using a modified PCR-based method (114) (Figure 5). Primers used to create the linker-scanning mutants are listed in Table I. The A amplicon was generated using two primers: Amain and An. Amain is a gene-specific sense primer that is homologous to -327 and -309 regions of the eNOS promoter. An, where n corresponds to the location of the specific 10 bp to be mutated, is a 25-mer, antisense primer containing a 15 bp sequence homologous to the eNOS promoter and a 10 bp mutation at the 5'-end that is heterologous to eNOS sequence. Similarly, the B amplicon was generated using two primers: Bmain and Bn. Bn is a 25mer, sense primer containing 15 bp of homologous eNOS sequence and a heterologous 10 bp mutation at the 5'-end of the primer. Bmain is a 21-mer homologous to the luciferase reporter gene sequence in the pGL2-Basic vector. All primer pairs were synthesized using a Beckman Oligo 1000 DNA synthesizer (Beckman Instruments Inc., Fullerton, CA) (Table I).

The 10 bp mutation of the An primer contains a Bgl II restriction endonuclease site (5'- A GATCT -3') and the 10 bp mutation of the Bn primer contains a BamHI restriction site [5'- G GATCC -3']. Therefore, 12 A amplicons and 12 B amplicons were generated by PCR and subsequently subcloned into the pCR II vector using the TA Cloning Kit from Invitrogen Corporation (San Diego, CA).
Figure 4. eNOS linker-scanning mutants. Linker-scanning mutants systematically introduced a 10 bp substitution, 5'-GCAGATCCGC-3', at positions indicated by shaded boxes.
Figure 5. Schemata of the PCR-based procedure used to generate the 10 bp linker-scanning mutants.
Table I. Primer pairs used to construct all pGL2-1193/+109 linker-scanning mutants.

<table>
<thead>
<tr>
<th>NAME</th>
<th>PRIMER SEQUENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>A main</td>
<td>5’-CCT TTA TGA CCC CCT GGT G -3’</td>
</tr>
<tr>
<td>B main</td>
<td>5’-TTT ATG TTT TTA GCC TCT TCC -3’</td>
</tr>
<tr>
<td>A 130+155</td>
<td>5’-CGA GAT CTG CAC GCC GCC TGG CCT G -3’</td>
</tr>
<tr>
<td>B 130+155</td>
<td>5’-CGG GAT CCG CTT TTA GAG CCT CCC A -3’</td>
</tr>
<tr>
<td>A 130+159</td>
<td>5’-CGA GAT CTG CCC TCA GCT CGA CGC C -3’</td>
</tr>
<tr>
<td>B 130+159</td>
<td>5’-CGG GAT CCG CTC CGA GCC GGG CTT G -3’</td>
</tr>
<tr>
<td>A 144+185</td>
<td>5’-CGA GAT CTG CGG CTC TAA AGC CTG A -3’</td>
</tr>
<tr>
<td>B 144+185</td>
<td>5’-CGG GAT CCG CGG TTC TGG CTG TCC C -3’</td>
</tr>
<tr>
<td>A 130+125</td>
<td>5’-CGA GAT CTG CCC GCC TGG GAG GCT C -3’</td>
</tr>
<tr>
<td>B 130+125</td>
<td>5’-CGG GAT CCG CTT CGG ATT GTG TAT G -3’</td>
</tr>
<tr>
<td>A 120+125</td>
<td>5’-CGA GAT CTG CAG GAA CAA GCC CGG C -3’</td>
</tr>
<tr>
<td>B 120+125</td>
<td>5’-CGG GAT CCG CTT ATG GGA TAG GGG C -3’</td>
</tr>
<tr>
<td>A 110+145</td>
<td>5’-CGA GAT CTG CAC AAT GGG ACA GGA A -3’</td>
</tr>
<tr>
<td>B 110+145</td>
<td>5’-CGG GAT CCG CGG GCC GGG GGG AGG G -3’</td>
</tr>
<tr>
<td>A 104+135</td>
<td>5’-CGA GAT CTG CTG TCC CAT ACA CAA T -3’</td>
</tr>
<tr>
<td>B 104+135</td>
<td>5’-CGG GAT CCG CGG GGC GCA CTA CGT G -3’</td>
</tr>
<tr>
<td>A 64+45</td>
<td>5’-CGA GAT CTG CGG CCC GCC CCT ATC C -3’</td>
</tr>
<tr>
<td>B 64+45</td>
<td>5’-CGG GAT CCG CGG TGC AGA GCC CCC T -3’</td>
</tr>
<tr>
<td>A 58+75</td>
<td>5’-CGA GAT CTG CCC TGG CCC TCG CCC C -3’</td>
</tr>
<tr>
<td>B 58+75</td>
<td>5’-CGG GAT CCG CCC CCT CCC ACT GCC C -3’</td>
</tr>
<tr>
<td>A 46+95</td>
<td>5’-CGA GAT CTG CGC TCT CCA GTG CTG G -3’</td>
</tr>
<tr>
<td>B 46+95</td>
<td>5’-CGG GAT CCG CTC CTG CCT CTT G -3’</td>
</tr>
<tr>
<td>A 44+95</td>
<td>5’-CGA GAT CTG CTC GGG AGG GGG CTG T -3’</td>
</tr>
<tr>
<td>B 44+95</td>
<td>5’-CGG GAT CCG CTG GTC CCC TCC C -3’</td>
</tr>
<tr>
<td>A 56+123</td>
<td>5’-CGA GAT CTG CGG AGG GGG CAG TGG G -3’</td>
</tr>
<tr>
<td>B 56+123</td>
<td>5’-CGG GAT CCG CCT CCC TCT TCC TAA G -3’</td>
</tr>
</tbody>
</table>
Vectors containing inserts were identified by performing an analytical digest with EcoRI and electrophoresis. In order to determine the orientation of the A and B inserts, to confirm the incorporation of the mutation and to exclude that a PCR-associated point mutation was incorporated, the clones were sequenced using an automated ABI Prism 377 DNA sequencer (Perkin-Elmer Applied Biosystems Canada Inc., Mississauga, Ont.). The primer used was M13 Reverse (see oligonucleotides). Medi-scale DNA preparations of each verified A and B clone was then performed (Qiagen Inc., Chatsworth, CA).

The A fragment was generated by digesting the pCR II vector with Pst I and Bgl II, followed by isolating the fragment by preparative gel electrophoresis. Similarly, the B fragment was generated by digesting the pCR II vector with Bam HI and Bgl II, followed by isolating the fragment by preparative gel electrophoresis. These two fragments were then subcloned into the Pst I and Bgl II sites of the -1193/+109 pGL2 promoter / luciferase construct. Since Bam HI and Bgl II 5'-overhangs created by restriction endonuclease digestion are compatible, the B fragment can be cloned into the vector in either orientation. Thus, the positive clones were identified by performing mini-preparative plasmid DNA analysis, followed by a Pst I/Bgl II and Bst YI digestion (Figure 6). A new Bst YI restriction site is created when a Bam HI overhang is ligated to a Bgl II overhang.

\[
\begin{align*}
5' & \quad \text{-----GCA} \\ 
3' & \quad \text{-----CGTCTAG} \\
\text{Bgl II} & \quad \text{Bam HI} \\
5' & \quad \text{-----GCA\text{GATCCGC}-----} \\
3' & \quad \text{-----CGTCTAG\text{GC\text{G}------}}} \\
\text{Bst YI} & 
\end{align*}
\]
Figure 6. Gel electrophoresis of pGL2-1193/+109 linker-scanning mutant constructs cleaved with *Bst* YI. Lane 1 represents 1 kb ladder, lane 2 represents the wild-type pGL2-1193/+109 construct, lanes 3-14 represent -164/-155mut linker-scanning mutant constructs. DNA was analyzed by performing a *Bst* YI digestion, followed by gel electrophoresis on a 2.7% NuSieve GTG agarose gel (Mandel).
To confirm the incorporation of the final 10 bp linker mutation in the pGL2 -1193/+109 promoter / luciferase construct, the positive clones were once again sequenced using the automated ABI Prism 377 DNA sequencer. In turn, each positive clone was purified using a CsCl "double banding" plasmid purification procedure to provide transfection-grade DNA. The success of a particular gene transfer experiment depends largely on the quality of the donor DNA preparation. In particular, there is a requirement for highly purified form I (covalently closed, circular supercoiled) plasmid molecules, in order to ensure both consistency and optimal levels of gene expression in transient transfection assays.

Ultimately, a 10 base linker scanning mutation, with sequence [5'-GCAGATCCGC-3'], is created. This linker mutation replaces 10 bp of eNOS sequence within the region contained between bp -164 and bp -35 of the eNOS promoter region. Also, this 10 bp substituted mutation has no known cis-regulatory elements. Therefore, its substitution into the eNOS sequence will only reflect the effect of any important cis-DNA elements already present in the native eNOS sequence. Ultimately, there is an exchange of wild type DNA sequences with heterologous sequences and in this way the topological and spatial relationships of the DNA helix are maintained (114).

-265/+109 linker mutants. The same 10 bp linker-scanning mutations were also incorporated into the pGL2 -265/+109 constructs. Each pGL2 -1193/+109 linker mutant construct was subjected to the following: The construct was cleaved in the multiple cloning site of the pGL2 -1193/+109 vector with Smal I. Simultaneously, the vector was cleaved with Pst I (which cleaves at -265, relative to the start site of transcription). Klenow enzyme was subsequently used to fill in the 3' overhang of the Pst I cut and a blunt-end ligation was performed. The positive clones were
identified by performing mini-preparative DNA isolation, followed by a Bgl II/Sal I and a Bst YI digestion, and by sequencing using the automated ABI Prism 377 DNA sequencer. The primer used was PM 833B (see Primers and Oligonucleotides). The -124/-115mut construct contains a novel Pst I site which was created with the incorporation of the 10 bp mutation. Therefore, a partial Pst I digestion was necessary to create the corresponding linker mutant in the context of the pGL2 -265/+109 construct.

Transfection-grade DNA of all -265/+109 linker-scanning constructs were obtained and this will be followed by transient transfection of bovine aortic endothelial cells. By comparing the luciferase activities of each linker mutant construct in the pGL2 -1193/+109 construct, the regulatory cis-elements necessary for functional promoter activity can be assessed. For example, if a pGL2 -265/+109 linker mutant construct exhibited low luciferase activity, when compared to the maximally active, wild type -265/+109 construct, then this may suggest that this 10 bp region contains a positive cis-regulatory element necessary for functional core promoter activity. However, if the same linker mutation, now in the context of the -1193/+109 construct, exhibited a higher luciferase activity, then this may suggest that the important cis-regulatory elements present in this region may only play a major role in the proximal promoter rather than in the context of the whole promoter. Such approaches will allow large domain interactions in the native promoter to be defined.

**Transient Transfection Assays.** All transient transfections were carried out using the Lipofectin Reagent (Gibco BRL). Lipofectin Reagent is a preparation of cationic liposomes composed of a novel positively charged lipid DOTMA (N[1-(2,3-dioleyloxy)propyl] - N,N,N-trimethylammonium) and DOPE (dioleoyl phosphatidyl ethanolamine) (86). The cationic
vesicles containing DOTMA interact spontaneously and rapidly with polyanions such as DNA and RNA, resulting in liposome/polynucleotide complexes that capture 100% of the polynucleotide. The resulting polycationic complexes are taken up by cells following interaction at the anionic cell surface.

BAEC culture were grown on 60 mm dishes, in 3.5 ml of BAEC medium, at a seeding efficiency of 3.3 x 10^4 cells/ml, approximately 48 hours prior to the time of transfection. A Brightline Hemacytometer (VWR Scientific, Toronto, Ont.) was used to quantitate the plating density.

Transfection conditions were optimized using the SV40 promoter / enhancer luciferase control plasmid (pGL2-Control). The amount of Lipofectin reagent together with the amount of DNA used was also optimized.

The plasmid DNA and Lipofectin Reagent were each diluted in 750 µl of OPTI-MEM I Reduced Serum Medium (Gibco BRL), which was equilibrated at room temperature. A 2:1 (mass:mass) Lipofection:DNA ratio was used to carry out the transfection procedure. The Lipofectin / OPTI-MEM I solution was incubated at room temperature for 30 minutes. The endothelial cells were co-transfected with 1.0 µg of linker mutant construct or wild type construct, 0.5 µg of pRSV-β-gal and 1.5 µg of pBluescript II SK (-) DNA. β-galactosidase activity was used to control for transfection efficiency from dish to dish and pBluescript II SK(-) DNA was used to increase transfection efficiency overall. The two OPTI-MEM I mixtures were combined to make a total of 1.5 ml and incubated at room temperature for 60 minutes, which allowed for the formation of DNA / Lipofectin complexes. In the meantime, the BAECs were washed with non-serum containing medium, OPTI-MEM I or RPMI 1640. Nine milliliters of OPTI-MEM I were
added to DNA / lipid solution, pipetted up and down 3 times and then overlaid on the endothelial cell culture. The cells were maintained at 37 °C, at 5% CO₂ for 5 hours. After incubation the transfection mix was replaced with RPMI 1640, supplemented with 15% bovine serum and antibiotics. Each experimental dish was performed in triplicate and each transfection experiment was performed at least three times.

The pGL2-Control vector contains the SV40 promoter and enhancer sequences, resulting in strong luciferase expression in many mammalian cell types. Therefore, this plasmid was used to monitor transfection efficiency and as a positive control for the promoter activities expressed by the eNOS pGL2 recombinants. The pGL2-Basic vector lacks an eukaryotic promoter and enhancer sequences. Thus, this plasmid was used as a negative control for the promoter activities expressed by the eNOS pGL2 recombinants.

Transfections performed with non-endothelial cell types (Table II) were conducted in a similar manner as stated above, except that 5 μg of experimental plasmid and 1 μg of pRSV-β-gal were used, together with 6 μg of Lipofectin reagent. HepG2 and Cos7 studies were performed in a similar fashion to the endothelial cell studies, except that HepG2 cells were seeded at a concentration of 5 x 10⁵ cells/ml and Cos7 cells were seeded at a concentration of 0.5 x 10⁵ cells/ml.

Protein Extraction. 48 hours after transfection, cells were harvested for protein extraction.

BAEC culture was washed once with Hank's Balanced Salt Solution without Mg²⁺ or Ca²⁺ (Gibco BRL) and then lysed with 300 μl of lysis buffer (0.1 M potassium phosphate buffer (pH 7.8), 1% Triton X-100, 1mM DTT, 2mM EDTA). Triton X-100 is a non-ionic detergent, which
solubilizes the luciferase enzyme; thus little enzyme remains bound to the cellular debris. The luciferase enzyme is most stable at a pH between 7.5 and 8.0 and thus a potassium buffer of pH 7.8 is used. 1 mM DTT is added to aid in the enzyme stability and 2 mM EDTA is added to chelate heavy metal ions which may interfere with luciferase activity. The lysis buffer was evenly distributed over the surface of the dish. The cell culture dishes, now containing 300 μl of lysis buffer were incubated on the Belly Dancer apparatus (BIO/CAN Scientific, Mississauga, Ont.) for 20 minutes, at 4 °C. Following lysis, the cells were scraped and collected into Eppendorf tubes. Protein extracts were centrifuged at 14000 rpm in a Beckman model centrifuge for 2 minutes to pellet residual cellular debris. The supernatants were then transferred to a fresh tube where they were either immediately assayed for luciferase activity or stored at -80 °C for subsequent assays that would assess β-galactosidase activity and protein concentration.

**Luciferase Assays.** Cell extracts were assayed for luciferase activity immediately after harvesting. Luciferase activity was measured using the MonoLight 2010C luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI).

The reporter gene luciferase encodes for an enzyme from firefly Photinus pyralis. Luciferase is a monomeric protein (62 kDa) that does not require any post-translational processing for enzymatic activity. Thus, it can function as a genetic reporter immediately upon translation. This enzyme catalyzes bioluminescence in the firefly by oxidizing D-luciferin in the presence of ATP, Mg$^{2+}$ and O$_2$. Specifically, it catalyzes the following reactions: (72, 328)
Mg$^{2+}$

(1) \[ \text{LH}_2 + \text{CoA} + \text{ATP} + E \xrightarrow{\text{formation}} E - \text{LH}_2\text{AMP} + \text{PP}_i \]

(2) \[ E - \text{LH}_2\text{AMP} + \text{O}_2 \xrightarrow{\text{reaction}} E - \text{P} + \text{AMP} + \text{CO}_2 + \text{Light (562 nm)} \]

(3) \[ E + L + \text{ATP} \xrightarrow{\text{formation}} E - \text{LAMP} + \text{PP}_i \]

\[
\begin{align*}
\text{LH}_2 & \quad = \quad \text{D-luciferin} \\
E & \quad = \quad \text{Firefly luciferase} \\
L & \quad = \quad \text{Dehydroluciferin} \\
P & \quad = \quad \text{Oxyluciferin} \\
\text{ATP} & \quad = \quad \text{Adenosine triphosphate} \\
\text{AMP} & \quad = \quad \text{Adenosine monophosphate} \\
\text{PP}_i & \quad = \quad \text{Inorganic pyrophosphate} \\
\text{CoA} & \quad = \quad \text{Coenzyme A}
\end{align*}
\]

The first reaction involves the formation of an enzyme-bound luciferyl-adenylate complex and free inorganic pyrophosphate. The second reaction involves this enzyme-bound complex rapidly reacting with molecular oxygen to produce light, CO$_2$ and AMP, while forming oxyluciferin bound to the enzyme. The level of light emitted during the reaction is measured by the luminometer. The third reaction summarizes an alternate pathway for the luciferase enzyme. If luciferase reacts with dehydroluciferin (an analog of luciferin that cannot be oxidized and thus is a competitive inhibitor), this results in the formation of dehydroluciferyl-adenylate tightly bound to the enzyme and no light is produced.
To assay for luciferase activity 25 μl of protein extract is used. The synthetic substrate used for this assay is D-Luciferin (D(-)-2-(6'-hydroxy-2'-benzothiasolyl)-thiazoline-4-carboxylic acid). 100 μl of D-Luciferin (1 mM) and 100 μl of luciferase assay buffer (30 nM Tricine (Calbiochem. La Jolla, CA), 3mM ATP, 15 mM MgSO₄, 10mM DTT, 1mM Coenzyme A (Calbiochem. La Jolla, CA), pH 7.8) are automatically injected by the dual injection system of the luminometer. Coenzyme A is a compound used to sustain light output for an extended period of time (4). By affecting the luciferase kinetics, Coenzyme A reacts with the inhibitory oxyluciferin-enzyme complex to form free enzyme. AMP and oxyluciferyl-CoA. This ultimately results in the removal of the inhibitory complex and a faster turnover rate of enzyme. Measurements of light units were integrated over a 10-second period.

Data in raw luciferase units (RLU) is normalized for the non-specific background of mock-transfected cells, which represented = 0.5% of most experimental luciferase activities. Intra- and inter-assay coefficients of variation averaged 7% and 9 %, respectively.

\textit{β-Galactosidase Assay.} β-D-galactosidase is encoded for by the \textit{LacZ} gene of E. Coli. For efficient translation in eukaryotic cells the translation initiation site has been optimized. This activity is used as a measure of transfection efficiency from dish to dish. CPRG (chlorophenol red-β-D-galactopyranoside, C₂₅H₂₁O₁₀Cl₂SNa) is the chromogenic substrate used for β-D-galactosidase. This enzyme catalyzes the following reaction:

\[
\text{β-D-galactosidase} \\
\text{CPRG} + \text{H}_2\text{O} \rightarrow \text{chlorophenol red} + \text{D-galactose}
\]
40 μl of cell extract was mixed with 139 μl of 0.1 M sodium phosphate buffer (pH 7.3), 3.6 μl of 0.5 M MgCl₂, 16 μl of 0.1 M CPRG solution, and 1.4 μl of 14.3 M 2-mercaptoethanol. The final volume of the reaction mix was 200 μl. CPRG was dissolved in 0.1 M sodium phosphate buffer (pH 7.3), made fresh before each assay. The reaction mix was thoroughly vortexed, spun down and incubated at 37 °C for 20 minutes. A bright, ruby red colour appeared by the end of the incubation period. To stop the reactions, 500 μl of 1 M Na₂CO₃ is added to each tube. 1 M Na₂CO₃ was made fresh before each assay and it was used to adjust the pH of the reaction to approximately 11, stopping further enzymatic conversion of CPRG. The reaction mixtures were thoroughly mixed, spun down and transferred to disposable cuvettes (SARSTEDT, St. Laurent, Quebec) and immediately read for optical density at 570 nm.

Control reactions used in this assay include a positive and a negative control. As a negative control, 40 μl of mock-transfected cell extract was used. As a positive control, 40 μl of mock-transfected cell extract was used along with 1 μl of β-galactosidase (Sigma, 50 units/ml). The negative control will give a measure of the endogenous β-galactosidase activity and at the end of the incubation period the reaction mix is usually yellow in colour, whereas the positive control is bright, ruby red.

The β-galactosidase activity of the experimental dishes was corrected for background. This calculation permits the measurement of the β-galactosidase activity contributed by the transfected constructs themselves. The product of the calculation was then used in the normalization of the data.
**Measurement of Protein Concentration.** A Bio-Rad Protein Assay (Bradford) was performed to determine the concentration of solubilized protein in each dish. This procedure is a dye-binding assay in which a differential colour change of a dye occurs in response to various concentrations of protein. 25 μl of protein extract was diluted 10-fold in 225 μl of distilled water. The Bradford Reagent (Bio-Rad) was diluted 5-fold in distilled water and filtered through a 0.22 μm filter using a syringe. 2 ml of this acidic dye was then added to 40 μl of diluted cell extract, mixed by inversion, transferred to a disposable cuvette (SARSTEDT, St. Laurent, Quebec) and subsequently measured at an optical density of 595 nm with a Pharmacia LKB Ultraspec Plus spectrophotometer after a 5 minute incubation period. The absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 dye shifts from 465 nm to 595 nm when protein binding occurs. Coomassie blue dye binds primarily basic and aromatic amino acid residues, especially arginine.

The measurements are ultimately compared to a standard curve created with bovine serum albumin to provide a relative measurement of protein concentration. This assay is used to account for protein variability from dish to dish in each transfection experiment.

**DNA Analysis.** It is essential to accurately control for the amount and quality of transfected DNA used per dish given that studies are performed under conditions in which luciferase activity is directly proportional to the amount of transfected DNA. The concentration of maxi-preparative DNA was accurately determined using three methods. A DyNA Quant 200 Fluorometer (Hoefer Pharmacia Biotech Inc.), an Ultraspec Plus UV/Visible Spectrophotometer (Pharmacia Biotech, Uppsala, Sweden) and uncut DNA subjected to gel electrophoresis were used to accurately quantitate the DNA. DNA was also analyzed by performing a Bst YI
digestion, followed by gel electrophoresis on a 2.7% NuSieve GTG agarose gel (Mandel) (Figure 6). Uncut DNA was also subjected to gel electrophoresis on a 0.7% agarose gel (Gibco BRL) (Figure 7).

**Primers and Oligonucleotides.** All oligonucleotides were created using the Beckman Oligo 1000 DNA synthesizer (Beckman Instruments, Mississauga, Ontario). The primers used to create the -1193/+109 linker mutants are listed in Table 1. M13 Reverse [5'- AAC AGC TAT GAC CAT G -3'] was used as a primer to sequence the positive pCR II plasmid clones. This primer is homologous to sequences of the lacZα cassette of the pCR II vector. MDN 30-1 was the primer used to sequence the -1193/+109 linker mutants [sense strand: 5'- CCA CAT CAC AGA AGG ACC -3'] and this sequence is homologous to -343 to -326 of the eNOS promoter. PM 833B was the primer used to sequence the -265/+109 linker mutants [anti-sense strand: 5'- CGC AGG GTG GCC CAG GCT -3'] and this primer is homologous to +51 to +68 of the eNOS gene.

**Data Analysis.** Unless otherwise indicated, data are expressed as the mean ± S.E.M. obtained in at least three independent transfection experiments, each done in triplicate. Comparisons were made with analysis of variance (ANOVA), followed by the Student-Newman-Keuls test. The level of statistically significant difference was defined as p<0.05.
Figure 7. Gel electrophoresis of the pGL2-1193/+109 linker-scanning mutant constructs. Lane 1 represents supercoiled DNA ladder, lane 2 represents the wild-type pGL2-1193/+109 construct, lanes 3-14 represent -164/-155mut linker-scanning mutant constructs. Uncut DNA was subjected to gel electrophoresis on a 0.7% agarose gel (Gibco BRL).
II.4 RESULTS

II.4.1 TRANSIENT TRANSFECTION FUNCTIONAL STUDIES IN ENDOTHELIAL CELLS USING DELETION MUTANT CONSTRUCTS

II.4.1.1 Transient Expression Analysis of the Human eNOS Distal Deletion Mutant Constructs in BAEC

In an effort to analyze the mechanisms of transcriptional regulation important in the human eNOS gene, our laboratory has created a series of deletion promoter / reporter constructs using the 5' flanking region of the human eNOS gene (9, 156). Restriction and modifying enzymes were used to create a series of seven 5' to 3' deletions, thus generating eNOS promoter / reporter gene constructs using the promoterless luciferase gene located in the plasmid pGL2-Basic. All constructs shared a common 3' end, ending at +109 relative to the start site of transcription but varied 5' ends. These deletion constructs were then assayed for luciferase expression following transient transfections of bovine aortic endothelial cells (Figure 8). The construct pGL2 -1193/+109 exhibited maximal functional promoter activity, stronger activity than the longer constructs pGL2 -3500/+109 and pGL2 -1900/+109. This suggests the existence of a negative regulatory region active in endothelium upstream of -1193. Relative to the luciferase control plasmid (pGL2-Control), where the expression of the luciferase gene was directed by a heterologous SV40 promoter and enhancer, the activity of the construct pGL2 -1193/+109 averaged 10% to 20% ± 1% S.E.M. In these specific constructs, deletion of sequences downstream of -265 resulted in a dramatic drop in functional promoter activity.
Figure 8. Activity profile of distal human eNOS promoter/reporter luciferase 5'- to 3'- deletion constructs in BAEC. To control for transfection efficiency cells were co-transfected with pRSV-βgal and relative luciferase activity was normalized for protein and β-galactosidase values. The data from these transient transfections are expressed as % luciferase activity relative to pGL2-1193/+109 and represent the mean ± S.E.M. (3 independent experiments, triplicate determinations). The maximally active construct, pGL2-1193/+109, displayed 10% to 20% of the activity an SV40 promoter/enhancer-directed luciferase control vector, pGL2-Control, displayed. Where error bars are not evident S.E.M. is below the figure resolution.
II.4.1.2 Transient Expression Analysis of the Human eNOS Proximal Deletion Mutant Constructs in BAEC

In order to define the important cis-regulatory regions 3' of -265, a further series of transient transfections were carried out using six additional deletion constructs designed to examine the region downstream of -265 in further detail (Figure 9). Deletion of sequences from -265 to -151 produced an approximate 33% drop in total activity relative to the maximally active -1193/+109 construct. Once sequences between -151 and -133 were deleted, a further 33% drop in activity was observed. Deleting sequences downstream of -133 resulted in a final drop in activity. Deleting sequences downstream of -92 resulted in no changes in functional promoter activity. This suggested that two positive regulatory regions exist in the proximal core promoter, one between -151 and -133, and the other between -133 and -92. Taking these sets of experiments together, these findings indicate: i) that regions between -1193/+109 have maximal functional promoter activity in endothelial cells, and ii) that genomic regions encompassing -133/+109 represent the minimally active promoter construct, or core promoter, for basal eNOS transcription in vascular endothelium in vitro.

In an attempt to correlate these functional results with the presence of cis-acting DNA elements in the 5' flanking region, one concludes that a paucity of well recognized positive cis-regulatory elements are evident in the positive regulatory region located between -151 to -133. whereas a high-affinity Sp1 site is located in the positive regulatory region located between -133 to -92. Cis-acting DNA elements, located downstream of -92, that may play a role in basal and endothelial cell-specific eNOS gene transcription cannot be assessed with deletion constructs.
Figure 9. Activity profile of proximal human eNOS promoter/reporter luciferase 5’- to 3’- deletion constructs in BAEC. To control for transfection efficiency cells were co-transfected with pRSV-βgal and relative luciferase activity was normalized for protein and β-galactosidase values. The data from these transient transfections are expressed as % luciferase activity relative to pGL2-1193/+109 and represent the mean ± S.E.M. (3 independent experiments, triplicate determinations). The maximally active construct, pGL2-1193/+109, displayed 10% to 20% of the activity an SV40 promoter/enhancer-directed luciferase control vector, pGL2-Control, displayed. Where error bars are not evident S.E.M. is below the figure resolution.
For example, if there exists another important cis-acting element downstream of -92, further deletion constructs will not unveil this potential cis-element because functional promoter activity has been reduced to negligible levels. Thus, deletion of sequences 3' of -92 would not further affect promoter activity, since activity is already at a negligible level.

II.4.1.3 Transient Expression Analysis of the Human eNOS Deletion Mutant Constructs in HUVEC

Luciferase expression was also assayed following transient transfections of HUVEC and normalized using β-galactosidase activity and protein concentrations. Comparison of the functional promoter activity of a series of human eNOS promoter/reporter constructs in HUVEC (Figure 10) revealed qualitatively similar functional activity response profiles. This suggests that BAEC and HUVEC share in common a series of functionally relevant trans-acting factors necessary for basal expression of the eNOS promoter.

II.4.2 TRANSIENT TRANSFECTION FUNCTIONAL STUDIES IN ENDOTHELIAL CELLS USING LINKER-SCANNING MUTANT CONSTRUCTS

In order to obtain a more mechanistic understanding of the mechanisms implicated in the regulated expression of the eNOS gene in endothelial cells, a systematic mutational analysis of the proximal promoter region was conducted. Thus, if consecutive 10 base pair sequences were mutated across the 5' flanking region, then the requirement for these regions with respect to transcriptional regulation of eNOS can be analyzed. This process of creating consecutive "blocks" of mutated sequence across a DNA region is called linker-scanning mutagenesis.
Figure 10. Activity profile of human eNOS promoter/reporter luciferase 5'- to 3'- deletion constructs in HUVEC. To control for transfection efficiency cells were co-transfected with pRSV-βgal and relative luciferase activity was normalized for protein and β-galactosidase values. The data from these transient transfections are expressed as % luciferase activity relative to pGL2-1193/+109 and represent the mean ± S.E.M. (3 independent experiments, triplicate determinations). The maximally active construct, pGL2-1193/+109, displayed 10% to 20% of the activity an SV40 promoter/enhancer-directed luciferase control vector, pGL2-Control, displayed. Where error bars are not evident S.E.M. is below the figure resolution.
scanning mutagenesis is a powerful method with which to assay the importance of individual DNA sequence elements within a transcriptional control region. Ultimately, there is an exchange of wild type DNA sequences with heterologous sequences and in this way the topological and spatial relationships of the DNA helix are maintained (114). This detailed approach allows mapping of the cis-acting DNA elements that are required for functional activity of the eNOS promoter.

II.4.2.1 Transient Expression Analysis of the Human eNOS pGL2 -1193/+109 Linker-Scanning Mutant Constructs in BAEC

To define the functionally important cis-acting DNA elements in the human eNOS minimal core promoter, a systematic mutational analysis of the proximal promoter region of the eNOS gene was undertaken. Specifically, twelve 10 bp linker-scanning mutant constructs were created in the context of the maximally active pGL2 -1193/+109 promoter / luciferase construct and spanned a 120 bp region from –164 to –45 relative to the start site of transcription. These mutants are site-specific with each representing the same 10 bp sequence [5′- GCA GAT CCG C -3′]. Promoter / reporter expression of each linker-scanning mutant construct was assayed following transient transfections of BAEC (n=4, triplicate determinations) (Figure 11). Data are normalized for β-galactosidase activity and protein concentration. Though bacterial methylase recognition sites [GATC] and [CCWGG] are found in the proximal eNOS promoter, studies performed with plasmid DNA prepared in dam- and dcm- bacterial strains indicated no functional effects (data not shown). Multiple lots of DNA were assessed in each transfection and displayed similar results.
Figure 11. Activity profiles of human eNOS -1193/+109 promoter/reporter luciferase linker-scanning mutations in BAEC. Linker-scanning mutants introduced a 10 bp substitution [5'-GCAGATCCGC-3']. To control for transfection efficiency cells were co-transfected with pRSV-β-gal and relative luciferase activity was normalized for protein and β-galactosidase values. The data from these transient transfections are expressed as % luciferase activity relative to pGL2 -1193/+109 and represent the mean ± S.E.M. (3 independent experiments, triplicate determinations). The maximally active construct, pGL2-1193/+109, displayed 10% to 20% of the activity an SV40 promoter/enhancer-directed luciferase control vector, pGL2-control.
The -164/-155mut and -155/-145mut linker-scanning constructs did not demonstrate any significant change in luciferase activity compared to the wild type construct, pGL2 -1193/109. This data suggests that no important functional *cis*-DNA elements reside between -164 and -145 relative to the transcription start site. The -144/-135mut, -134/-125mut, and -124/-115mut linker-scanning constructs displayed a significant 40 - 50 % drop in functional promoter activity relative to the wild type construct (p< 0.0001). A 10 bp mutation at -114 to -105, had no functional effect on the activity of the eNOS promoter. A 10 bp mutation involving -104/-95 resulted in a profound 85% drop in luciferase activity compared to the wild type promoter construct, -1193/+109 (p< 0.0001). Mutating the sequences between -94 and -45, as consecutive 10 bp mutations, resulted in no significant change in functional promoter/reporter activity in vascular endothelial cells.

Taken together, four of the eNOS promoter / reporter constructs derived from the linker-scanning mutant series resulted in a significant decrease in luciferase expression. These constructs defined two regions of interest: -104/-95 (designated as positive regulatory domain I (PRD I)) and -144/-115 (PRD II). The PRD I linker mutant -104/-95mut has the most robust effect on transcription, showing an ~ 85% drop in activity. The linker mutants belonging to PRD II (-144/-135mut, -134/-125mut, and -124/-115mut) displayed a significant 40-50 % drop in luciferase activity as compared to the wild type pGL2 -1193/+109 construct. The existence of PRD I and II were initially suggested with deletion promoter / reporter constructs. With the linker-scanning mutagenesis approach, the PRD I and II domains were specifically localized to smaller regions of DNA sequence. Thus, this method may be used to further refine *cis*-regulatory DNA domains. Overall, this data suggests that all three domains contain positive regulatory elements that are important in the activation of the eNOS promoter.
II.4.2.2 Transient Expression Analysis of the Human eNOS pGL2 -265/+109 Linker-Scanning Mutant Constructs in BAEC

The same 10 bp linker-scanning mutations were also incorporated into the pGL2 -265/+109 construct. This construct contains a shorter eNOS 5' flanking region. A comparison between the activity profile demonstrated by the shorter promoter linker mutants with the activity profile demonstrated by the same 10 bp mutations in the context of a longer eNOS promoter region would allow large domain interactions to be defined and/or mapped. For example, if the cis-regulatory regions upstream of -265 are necessary for transactivation from PRD II, then a difference will exist in the effects of the varied mutants that span PRD II. Promoter / reporter expression of each linker-scanning mutant construct was assayed following transient transfections of BAEC (n=3, triplicate determinations) (Figure 12).

No significant change in activity, relative to wild type, was observed when the sequence between -154 and -145 was mutated. However, -144/-135, -134/-125, -124/-115 and -114/-105 mutations resulted in a significant ~30-40% drop in activity, relative to wild type (-144 (0.005<p<0.01), -134 (0.0001<p<0.005), -124 and -115 (p<0.0001)). On the other hand, the 10 bp mutation involving -104/-95 resulted in a profound 90% drop in luciferase activity, compared to the wild type promoter construct, -265/+109 (p<0.0001). Mutating the sequence between -94 and -45, as consecutive 10 bp mutations, resulted in no significant change in activity.

Taken together, both positive regulatory domains (PRD I and PRD II) are evident in the context of the shorter promoter/reporter construct. As in the -1193/+109 linker-scanning mutant profile,
Figure 12. Activity profiles of human eNOS -265/+109 promoter/reporter luciferase linker-scanning mutations in BAEC. Linker-scanning mutants introduced a 10 bp substitution [5'-GCAGATCCGC-3']. To control for transfection efficiency cells were co-transfected with pRSV-β-gal and relative luciferase activity was normalized for protein and β-galactosidase values. The data from these transient transfections are expressed as % luciferase activity relative to pGL2 -265/+109 and represent the mean ± S.E.M. (3 independent experiments, triplicate determinations). The maximally active construct, pGL2-265/+109, displayed 3% of the activity an SV40 promoter/enhancer-directed luciferase control vector, pGL2-control. Where error bars are not evident the S.E.M. is below the figure resolution.
the -104/-95 mutation has the most robust effect, ~90% drop in activity. The PRD II mutants (-144/-135, -134/-125 and -124/-115) displayed a significant 30-40% drop in luciferase activity.

II.4.2.3 Transient Expression Analysis of the Positive Regulatory Domain Mutant Constructs

In order to directly compare the effects of PRD I and PRD II linker-scanning mutations in the context of the longer (pGL2 -1193/+109) and shorter (pGL2 -265/+109) promoter/reporter constructs, a transient transfection assay in BAECs was performed using the constructs that correspond to these positive regulatory domains (Figure 13).

In both contexts, a PRD I mutation, -104/-95, resulted in an ~90% drop in functional promoter activity, relative to the wild type constructs. The mutations that account for PRD II, -144/-135, -134/-125 and -124/-115, demonstrated an ~30-40% drop in luciferase activity in both the pGL2 -1193/+109 and pGL2 -265/+109 constructs.

Taken together, functional reporter activities revealed no important differences compared to linker-scanning mutations in the setting of the maximally active -1193/+109 construct. A synopsis of this data is presented in Figure 13. These data are taken to indicate that functional contributions from PRD I and II are not dependent upon additional sequences present between -1193 and -265 (n=3, triplicate determinations).
Figure 13. Comparison of promoter activity of positive regulatory domain I (PRD I) and positive regulatory domain II (PRD II). To control for transfection efficiency cells were co-transfected with pRSV-βgal and relative luciferase activity was normalized for protein and β-galactosidase values. The data from these transient transfections are expressed as % luciferase activity relative to pGL2-1193/+109 and represent the mean ± S.E.M. (3 independent experiments, triplicate determinations). The maximally active construct, pGL2-1193/+109, displayed 10% to 20% of the activity an SV40 promoter/enhancer-directed luciferase control vector, pGL2-Control, displayed. Where error bars are not evident S.E.M. is below the figure resolution.
II.4.3 TRANSIENT TRANSFECTION FUNCTIONAL STUDIES IN NON-ENDOTHELIAL CELL TYPES

To determine whether the 5'-flanking sequences of eNOS direct endothelial cell-specific expression, selected promoter/reporter constructs have been analyzed in cell lines that do not express native eNOS mRNA transcripts as assessed with Northern blot and RNase protection analyses (data not shown and Figure 14A). The pGL2-1193/+109 and pGL2-133/+109 constructs were transiently transfected into a number of endothelial and non-endothelial cells (Table IIa and IIc). As discussed above, the pGL2-1193/+109 construct was the maximally active construct in both HUVEC and BAEC. As predicted, the promoter/reporter construct exhibited strong activity in endothelial cells compared to another endothelial cell-restricted gene, preproendothelin-1 (ET-1): a strong heterologous promoter, the SV40 promoter/enhancer: and a weak heterologous promoter, the thymidine kinase promoter (Table IIb). Surprisingly, 60% of the non-endothelial cell lines used in this analysis displayed trivial eNOS promoter activity (pGL2-1193/+109 construct), but the other 40% displayed an unusually high promoter activity (see Table IIc). These include HepG2, TGW, KPN, F9 and P19. The pGL2-133/+109 construct was found to be broadly active in all cell-types except for the Schneider Drosophila (SL2) cell line. HepG2 cells do not normally express the native eNOS transcript, however, this human hepatocellular carcinoma cell line exhibited high promoter activity with the -1193 construct and low activity with the -133 construct. Under baseline conditions, the native eNOS gene is not expressed in this cell line (Figure 14A). A similar finding was also found with the F9, TGW, KPN, P19 and NIH 3T3 transfections.
Figure 14A. eNOS mRNA expression in endothelial and non-endothelial cells. Lane 1 is the molecular weight size standard. (-) indicates that no RNase was added to the cells; (+) indicates the addition of RNase. The protected fragment is 292 nt in length and the probe is 340 nt.
Table IIa. Activity of the human eNOS promoter in varied cell types.

<table>
<thead>
<tr>
<th>CONSTRUCTS</th>
<th>pGL2-133/+109</th>
<th>pGL2-1193/+109</th>
<th>pGL2-CONTROL</th>
<th>(-133/-1193)x100</th>
<th>pGL2-133/+109</th>
<th>pGL2-1193/+109</th>
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<tr>
<td>CELL TYPES</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BAEC</td>
<td>2890 ± 419</td>
<td>11300 ± 1140</td>
<td>115000 ± 1500</td>
<td>25.6</td>
<td>2.5 ± 0.1</td>
<td>9.8 ± 0.4</td>
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<tr>
<td>HUVEC</td>
<td>514 ± 107</td>
<td>2180 ± 168</td>
<td>18900 ± 1530</td>
<td>23.6</td>
<td>2.7 ± 0.6</td>
<td>11.5 ± 0.9</td>
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<tr>
<td>Non-endothelial</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DAMI</td>
<td>1209 ± 27</td>
<td>483 ± 52</td>
<td>11200 ± 2600</td>
<td>26.4</td>
<td>2.7 ± 0.6</td>
<td>10.0 ± 0.3</td>
</tr>
<tr>
<td>HeLa</td>
<td>57 ± 18</td>
<td>212 ± 37</td>
<td>3260 ± 823</td>
<td>26.9</td>
<td>1.7 ± 0.5</td>
<td>6.5 ± 1.2</td>
</tr>
<tr>
<td>HepG2</td>
<td>493 ± 22</td>
<td>966 ± 54</td>
<td>13800 ± 3010</td>
<td>51.1</td>
<td>3.6 ± 0.2</td>
<td>7.0 ± 0.4</td>
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<tr>
<td>JEG-3</td>
<td>18 ± 5</td>
<td>54 ± 3</td>
<td>2690 ± 215</td>
<td>33.3</td>
<td>0.7 ± 0.2</td>
<td>2.0 ± 0.1</td>
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<tr>
<td>Jurkat</td>
<td>10 ± 1</td>
<td>16 ± 13</td>
<td>2780 ± 277</td>
<td>62.5</td>
<td>1.0 ± 0.6</td>
<td>0.3 ± 0.2</td>
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<td>KPN</td>
<td>949 ± 260</td>
<td>1540 ± 426</td>
<td>685 ± 112</td>
<td>61.6</td>
<td>140 ± 27</td>
<td>220 ± 44</td>
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<td>739 ± 48</td>
<td>178 ± 20</td>
<td>25.6</td>
<td>104 ± 18</td>
<td>420 ± 27</td>
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<td>F9</td>
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<td>5090 ± 450</td>
<td>14800 ± 310</td>
<td>2.0</td>
<td>0.7 ± 0</td>
<td>34.3 ± 3.0</td>
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<td>P19</td>
<td>807 ± 36</td>
<td>5760 ± 1030</td>
<td>12000 ± 3440</td>
<td>14.0</td>
<td>6.7 ± 0.3</td>
<td>48.0 ± 8.6</td>
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<td>NIH 3T3</td>
<td>58 ± 18</td>
<td>110 ± 34</td>
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<td>233000 ± 23500</td>
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<td>5.9 ± 0.7</td>
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* nd = not detected
Table IIb. Activity of the human preproendothelin-1 promoter in varied cell types.

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<th>ptk-GH (ng/ml)</th>
<th>% of ptk-GH (ng/ml)</th>
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<td>0.70 ± 0.06</td>
<td>0.05 ± 0.01</td>
<td>1310 ± 110</td>
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<td>HUVEC</td>
<td>116 ± 11</td>
<td>8.1 ± 1.3</td>
<td>1430 ± 140</td>
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<tr>
<td>Non-Endothelial</td>
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<tr>
<td>HeLa</td>
<td>11.2 ± 0.60</td>
<td>2.29 ± 0.60</td>
<td>491 ± 105</td>
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<td>HepG2</td>
<td>0.02 ± 0.01</td>
<td>0.09 ± 0.02</td>
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<td>Jurkat</td>
<td>0.12 ± 0.09</td>
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* nd = not detected
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<th>CELL TYPE</th>
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<td>Non-Endothelial Cell Types</td>
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<td>Human Squamous Cell Carcinoma Cell Line</td>
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<td>Human Hepatocellular Carcinoma Cell Line</td>
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<td>Human Choriocarcinoma Cell Line</td>
<td>+/-</td>
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<td>Jurkat</td>
<td>Human Mature T-cells</td>
<td>+/-</td>
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<td>KPN</td>
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</table>
II.4.3.1 Transient Expression Analysis of the 5'-Flanking Region in HepG2 cells.

In order to define the cell-specificity of these constructs a selection of eNOS promoter / luciferase deletion constructs were transiently transfected into a hepatocellular carcinoma cell line HepG2 (Figure 14B). The trends in activity were not similar to those observed in BAEC (Figures 8 and 9). The construct pGL2 -1193/+109 did not have maximal functional promoter activity as in the case of the BAECs. In contrast, the pGL2 -3500/+109 construct displayed maximal functional promoter activity. Its activity averaged 9% ± 0.4% (mean ± S.E.M.) of the activity of the luciferase control plasmid (pGL2-Control) whose expression was directed by the SV40 promoter and enhancer. Deletion of human eNOS sequences between -3500 and -1193 resulted in a significant ~30% drop in activity, suggesting that these sequences contain a positive regulatory sequence in HepG2 cells. Deletion of sequences from -1193 to -743 also resulted in a significant change in activity, approximately 80% decrease relative to the -1193/+109 construct. Deleting sequence from -151 to -133 also resulted in a slight decrease in functional promoter activity. Once sequences between -133 and -92 were deleted, a final drop in activity was observed. This latter deletion construct result is consistent with the BAEC activity profile.

II.4.3.2 Transient Expression Analysis of the 5'-flanking Region in Cos7 Cells

Preliminary transfection studies performed in Cos7 cells revealed unusual promoter activities for the pGL2-1193/+109 and pGL2-133/+109 constructs (data not shown). Therefore, a selection of deletion constructs were also transiently transfected into Cos7 cells (Figure 15). Cos7 cells are African green monkey kidney cells that have been transformed with SV40 large T antigen. The construct pGL2 -1193/+109 had demonstrated an activity between 2% ± 0.5% S.E.M. of the
Figure 14B. Activity profile of human eNOS promoter/reporter luciferase 5' to 3'-deletion constructs in HepG2. To control for transfection efficiency cells were co-transfected with pRSV-β-gal and relative luciferase activity was normalized for protein and β-galactosidase values. The data from these transient transfections are expressed as % luciferase activity relative to pGL2-1193/+109 and represent the mean ± S.E.M. (3 independent experiments, triplicate determinations). The maximally active construct, pGL2-3500/+109, displayed 9% of the activity an SV40 promoter/enhancer-directed luciferase control vector, pGL2-control. Where error bars are not evident S.E.M. is below the figure resolution.
Figure 15. Activity profile of human eNOS promoter/reporter luciferase 5' to 3'-deletion constructs in Cos7. To control for transfection efficiency cells were co-transfected with pRSV-β-gal and relative luciferase activity was normalized for protein and β-galactosidase values. The data from these transient transfections are expressed as % luciferase activity relative to pGL2-1193/+109 and represent the mean ± S.E.M. (3 independent experiments, triplicate determinations). The maximally active construct, pGL2-1193/+109, displayed 2% of the activity an SV40 promoter/enhancer-directed luciferase control vector, pGL2-control. Where error bars are not evident S.E.M. is below the figure resolution.
activity of the pGL2-Control construct. Compared to the relative activity of \textasciitilde{}1193/+109 in BAEC (10% ± 1%). this construct is relatively inactive. The construct pGL2 -3500/+109, which contains longer 5'- flanking sequences had a significantly lower activity than pGL2 -1193/+109 (approximately 10% of the activity of pGL2 -1193/+109). This observation suggests the possible presence of cis-regulatory DNA sequences, which exhibit a negative functional effect in Cos7 cells, between -3500 and -1193. A similar trend is also evident in BAECs (see Figure 8). This profile contrasts with results obtained in the HepG2 cell line (see Figure 14B). Deleting sequences between -1193 and -743 resulted in a dramatic drop in activity, suggesting the presence of a positive regulatory element present in those sequences. The most interesting finding came with deletions spanning the regions -151 to -133. A dramatic increase in functional promoter activity was observed. This implies that a negative regulatory element in Cos7 was removed. In contrast to the BAEC profile, the pGL2 -133/+109 construct displayed comparable luciferase activity to that of the pGL2 -1193/+109 construct. A final drop in activity is observed when the sequences between -133 and -92 were deleted. This trend is also seen with the BAECs.
II.5 DISCUSSION

The transient transfection analysis of the deletion studies have indicated: i) that regions between -1193/+109 have maximal functional promoter activity in endothelial cells, and ii) that genomic regions encompassing -133/+109 represent the minimally active promoter construct, or core promoter, for basal eNOS transcription in vascular endothelium *in vitro*. The 5'-flanking region assessed in these studies was demonstrated to be a promoter in a variety of endothelial cell types, including BAEC and HUVEC. The maximally active construct, pGL2-1193/+109, exhibited approximately 10 – 20 % promoter activity relative to the SV40 promoter/enhancer.

Two positive regulatory domains (PRD I and II), active in endothelial cells, have been identified in the minimal core promoter of the human eNOS gene using deletion and linker-scanning mutagenesis. PRD I was initially uncovered with deletion analysis in regions between -133 and -92, relative to the start site of transcription. Using the linker-scanning approach, the location of PRD I was refined to a 10 bp region (-104/-95mut construct). This linker mutation had the most profound effect on luciferase activity and corresponds to a high-affinity Sp1 cis-acting DNA element [5'- GGG GCG GGG C -3'] located between -104 and -95, relative to transcription initiation. Future nucleoprotein analysis will assess whether Sp1 family members play a functionally important role in PRD I. We found that the GATA cis-DNA element was not relevant in our studies. This is in contrast to previous work suggesting an important role of GATA in eNOS transcriptional regulation (359).

PRD II was also identified with the eNOS deletion construct analysis. A functional PRD II domain was defined between -151 and -133. Linker-scanning mutational analysis revealed that
PRD II encompasses a 30 bp region from −144 to −115. This positive regulatory region was uncovered by three linker-scanning constructs, -144/-135mut, -134/-125mut and -124/-115mut. Sequence inspection of this 30 bp reveals many putative cis-DNA elements. For example, a low-affinity Sp1 site [5′- GGG AGG -3′] is located on the anti-sense strand between −146 and −141. A p53 half-site [5′- GGG CTT GTT C -3′] is located on the sense strand between −136 and −127. The pentamer, [5′- GTT CC -3′] that is located at −119 to −125 is recognized by the paired domain of PAX family proteins. In addition, there is an Ets-like binding site, [5′- G GAA-3′], at position −129 to −126, located on the anti-sense strand. Finally, there is YY1-like element spanning the sequence from −121 to −117. The possibility that Sp1, p53, PAX, Ets and YY1 proteins contribute to the dynamics of the control for basal eNOS promoter activity needs to be addressed. Overall, it is clear that further functional and EMSA studies are necessary to identify the functional properties of the trans-acting factors interacting with the PRD II domain in vascular endothelium.

Linker-scanning mutagenesis is the process of creating consecutive "blocks" of mutated sequence across a DNA region. Linker-scanning mutagenesis is a powerful method with which to assay the importance of individual DNA sequence elements within a transcriptional control region. Ultimately, there is an exchange of wild type DNA sequences with heterologous sequences and in this way the topological and spatial relationships of the DNA helix are maintained. This detailed approach allowed the precise mapping of the cis-acting DNA elements with a better resolution compared to deletion mapping of a promoter. This method identified requirement of PRD I and II for the transcriptional regulation of the human eNOS gene in vascular endothelial cells.
Although these studies addressed the basal expression of eNOS, the inducible expression of eNOS was not studied. Future studies involving models of endothelial activation (eg. cytokines, shear stress) will address the effect on the transcriptional regulation of eNOS promoter activity.

An intriguing aspect of eNOS gene expression is its relative restriction to the vascular endothelium. Possible transcriptional regulatory mechanisms involved were examined through a series of transient transfection assays with eNOS promoter/reporter constructs in a variety of endothelial and non-endothelial cells. The pGL2-1193/+109 promoter/reporter construct was found to be maximally active in both HUVEC and BAEC compared to the other non-endothelial cell types (Tables IIa, IIb and IIc). Interestingly, this construct was also found to be quite active in a number of non-endothelial cell types in which no appreciable eNOS mRNA transcripts were detected by RNase protection assays and northern blot analysis (data not shown and Figure 14A). Taken together, these results suggest that these cells must possess some endogenous cell-specific mechanisms that actively repress native eNOS gene expression. For example, the HepG2 cell line does not express appreciable quantities of steady state eNOS mRNA, as assessed by northern blot analysis. In the HepG2 cell line, the high promoter activity evident with the -1193/+109 construct may suggest that cis-regulatory DNA elements act as cell-specific negative transcriptional control regions. These negative regulatory elements would therefore not be present in the specific episomal vectors used in these studies. Such a model has been proposed for the SCG10 gene expressed exclusively in neurons. This gene contains a neuron-restrictive silencer element (NRSE) which is recognized by a neuron-restrictive silencer factor (NRSF) (278). NRSF represses expression of neuron-specific genes in non-neuronal cell types by binding to the NRSE. Alternatively, this may suggest that the expression of eNOS may be controlled by means other than transcription such as: (i) transcriptional attenuation. where
transcription elongation is blocked in the proximal regions of the native promoter in non-endothelial cells, as in c-myc (25) and adenosine deaminase (158), resulting in an inability to generate full-length eNOS transcripts. (ii) epigenetic regulation, where the DNA methylation pattern of the native gene differs between endothelial and non-endothelial cell types, such that the episomal vector fails to recapitulate this status and is thus unable to mimic endogenous expression (27, 171) or (iii) post-transcriptional processes account for the absence of steady state eNOS mRNA levels in non-endothelial cells. For example, if the eNOS transcript has a long mRNA half-life in endothelial cells, and is labile in non-endothelial cells, this may present an alternate way of regulating eNOS expression.

Detailed transient transfections were also performed on HepG2 cells using a subset of the eNOS deletion promoter / luciferase constructs. Deletion studies revealed that a positive regulatory region exists between -3500 and -1193. This is in contrast to the BAEC profile, which displays a negative regulatory region spanning this area. Upon deleting sequences from -1193 to -743, a dramatic drop in activity was observed. In fact, the existence of PRD I and PRD II is also evident in the HepG2 profile as shown with the decrease in activity from -151/-133 and -133/-92. This suggests that the trans-acting factors interacting with PRD I and PRD II are not endothelial cell-specific and are also present in HepG2 cells.

A similar study was performed in Cos7 cells. Transfection studies performed in Cos7 cells revealed an unusual eNOS promoter/reporter expression profile. A negative regulatory region was evident between -151 and -133, previously shown as PRD II in endothelial cells. This gives rise to the possibility of PRD II is playing an essential role in the cell-specific expression of eNOS. The mechanism of this inhibition may be mediated by the T antigen, with which Cos7
cells are transformed. This may suggest that T antigen is modifying an important trans-acting factor(s) interacting with PRD II. For example, through phosphorylation, T antigen is known to modulate the biology of trans-regulatory eukaryotic transcription factors. An example includes p53, which is specifically phosphorylated by large T antigen (217). T antigen may also complex with that trans-acting factor(s) and hinder its binding capabilities to the DNA. It has been shown that SV40 large T antigen forms a stable complex with the retinoblastoma protein (69). Both these situations will result in a negative impact on the activation of the eNOS promoter. Another possibility may also be that an epithelial factor present in African green monkey kidney cells is responsible for this negative effect on PRD II. In other words, the composition of transcription factors in Cos7 cells may differ from endothelial cells and thus account for this difference in activity profile. Future studies involving PRD II analysis in Cos7 cells will elucidate the role of PRD II in eNOS cell-specific expression. In addition the study of the effect of SV40 large T antigen on the transcriptional regulation of the eNOS promoter will enable us to further dissect the role of PRD II in eNOS activation. This study will potentially identify the important trans-acting factors interacting with PRD II.

It is important to note that chromatin-based studies may be necessary to elucidate the mechanism of the cell-specific expression of the eNOS gene. The findings of the in vivo murine promoter/reporter insertional transgene work demonstrated that murine eNOS genomic regions spanning −5200/+28, relative to the transcription start site, direct expression of a reporter construct in a fashion that recapitulates the known expression profile of eNOS mRNA and protein. Therefore, a comparison of stable versus transient expression studies using −6000/+109 of human eNOS promoter sequence will allow the importance of chromatin structure in the cell-specific expression of the eNOS gene to be addressed.
CHAPTER III

CHARACTERIZATION OF THE HUMAN eNOS PROMOTER

(PART II)

Portions of this chapter have been published as:


CHAPTER III.  CHARACTERIZATION OF THE HUMAN eNOS PROMOTER (PART II)

III.1 OBJECTIVE AND AIMS

There exists a limited amount of knowledge with respect to transcriptional mechanisms regulating constitutive expression of endothelial cell-specific genes. A comprehensive understanding of the interdependent protein-DNA and protein-protein interactions that reciprocally interact with co-activators and the general transcription machinery on the native eNOS promoter is necessary for developing further insight into perturbations of eNOS expression in the diseased blood vessel wall. Based upon this premise, the present study focuses upon a detailed dissection of the multiprotein complexes that form upon functionally important cis-DNA elements and are implicated in the cooperative control of constitutive expression of the human eNOS gene in vascular endothelial cells. We posit that by studying the mechanisms underlying transcription of the eNOS gene further mechanistic insight into endothelial cell-specific gene expression may evolve.
III.1.1 OBJECTIVE

The overall objective of these studies is to examine the basic regulatory features implicated in the physiologic and pathophysiologic control of human eNOS gene transcription in vascular endothelial cells.

III.1.2 SPECIFIC AIMS

1. The first specific aim of this chapter is to identify the biologically important trans-acting factors that functionally interact with the cis-regulatory elements identified as Positive Regulatory Domain I and Positive Regulatory Domain II in vascular endothelial cells.

2. The second specific aim of this chapter is to characterize the functional importance of the above cis-DNA elements and corresponding trans-acting factors in the regulation of eNOS promoter activity in a heterologous expression system.

III.2 HYPOTHESIS

Multiprotein complexes form upon functionally important cis-DNA elements of the eNOS 5’-flanking region through protein-DNA and protein-protein interactions. These multiprotein complexes constitute the components of an enhanceosome that is implicated in the cooperative control of constitutive expression of the human eNOS gene in vascular endothelial cells.
III.3 MATERIALS AND METHODS

Materials. Cell culture media, balanced salt solutions, antibiotics and trypsin-EDTA were obtained from Gibco BRL (Grand Island, NY). Cell culture plates and Teflon scrapers were from Costar (Cambridge, MA). Restriction enzymes were from New England Biolabs (Beverly, MA) and Boehringer Mannheim (Mannheim, Germany). Anti-Sp1, anti-GATA, anti-Sp3, anti-Sp2, anti-Sp4 and anti-Ets-1/Ets-2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Protein assay kit was purchased from Bio-Rad (Hercules, CA). Cytidine 5'-[α-32P] tri-phosphate ([32P]dCTP; 3000 Ci/mmol) was from Dupont-New England Nuclear (Wilmington, DE). Cell culture grade bovine gelatin was purchased from Sigma Chemical (St. Louis, MO). CPRG (chlorophenol red-β-D-galactopyranoside) was purchased from Boehringer Mannheim (Mannheim, Germany). Coenzyme A and ATP were purchased from Calbiochem (La Jolla, CA). D-luciferin and luminometer cuvettes were purchased from Analytical Luminescence Laboratory/Pharmingen (Ann Arbor, MI). All luciferase assays were carried out on the MonoLight 2010 luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI). All optical density readings were performed on an Ultrospec Plus UV/Visible Spectrophotometer (Pharmacia Biotech, Uppsala, Sweden). Determination of DNA concentration was performed with a DyNA Quant 200 Fluorometer (Hoefer Pharmacia Biotech Inc., Uppsala, Sweden).

Cell Culture. Bovine aortic endothelial cells (BAEC) and SL2 (Drosophila Schneider's line 2) were propagated as described in Chapter II.

Plasmid Construction. Drosophila eukaryotic expression constructs. Expression cassettes for Sp1, Sp3 variants, Elf-1, Ets-1 and MAZ were based upon pPacUO, a transient episomal vector
which contains the 2.6 kb *Drosophila* actin 5C promoter, a 0.7 kb 5'-UTR Ultrabithorax (*Ubx*) internal ribosome entry site (IRES), the first eight codons of the *Ubx* open reading frame and 1.1 kb of 3'-UTR from the actin 5C gene. The latter provides polyadenylation signal sequences. pPacUSp1 and pPacUO was kindly provided by R. Tjian (Berkeley, IL) and has been described previously (28, 63). pPacUSp3 was provided by G. Suske (Marburg, Germany) (117). For construction of pPacUSp3ΔNH2, the pPacUSp3 plasmid was subjected to a partial *Vol* I and a *Bgl* II digestion. The 8.9 kb fragment was blunt-ended with Klenow and an intramolecular ligation performed removing 0.85 kb from the NH2-terminus of the Sp3 open reading frame leaving amino acids 297 to 667 of human Sp3 (Sp3ΔNH2). pPacUELf-1 was constructed using a full-length human Elf-1 cDNA kindly provided by J. M. Leiden (Chicago, IL). A 1.9 kb *Xho* I fragment was subcloned into the *Xho* I site of pPacUO. The resulting plasmid was subjected to a partial *Pst* I restriction digestion and blunt-ended with Klenow enzyme to remove 4 nt placing the Elf-1 cDNA in frame with the first 8 codons of the *Ubx* ORF. pPacUEts-1 was constructed using a full-length murine Ets-1 cDNA provided by B.J. Graves (Salt Lake City, UT). A 1.5 kb *Nde* I-*Bam* HI fragment was blunt-ended and subcloned into the blunt-ended *Bam* HI site of pPacUO. pCGN-MAZ was kindly provided by Thomas Shenk (Princeton, NJ) (242). For pPacUMAZ, pCGN-MAZ was cleaved with a partial *Eco*RI digestion. The linearized plasmid was blunt-ended and digested with *Bam*HI. pPacUO was cleaved with *Xho*I, blunt-ended and cleaved with *Bam*HI. The resulting 2.3 kb fragment containing the MAZ ORF was cloned into the *Bam*HI sites of pPacUO.

**Primers and Oligonucleotides.** All oligonucleotides were created using the Beckman Oligo 1000 DNA synthesizer (Beckman Instruments, Mississauga, Ontario). Oligonucleotides used in EMSA analysis are listed in Table III.
Table III. Oligonucleotides used in EMSA analysis.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5' - 3'; Sense Strand)</th>
</tr>
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<tbody>
<tr>
<td>-120/-91</td>
<td>CAT TGT GTA TGG GAT AGG GGC GGG GCG AGG</td>
</tr>
<tr>
<td>-120/-91mutSp1*</td>
<td>CAT TGT GTA TGG GAT AGG GCT CGG TCG AGG</td>
</tr>
<tr>
<td>-120/-91mutGATA'</td>
<td>CAT TGT GTA TGG AAT AGG GGC GGG GCG AGG</td>
</tr>
<tr>
<td>Sp1 (SV40 early promoter)</td>
<td>ATT CGA TCG GGG CGG GGC GAG</td>
</tr>
<tr>
<td>Ap2 (hMIIIa promoter)</td>
<td>GAT CGA ACT GAC CGC CCG CCC GT</td>
</tr>
<tr>
<td>-140/-111</td>
<td>TCC CAG CCG GGC TTG TTC TCC CAT TGT GTA T</td>
</tr>
<tr>
<td>-155/-120</td>
<td>GCT TTA GAG CCT CCC AGC CGG GCT TGT TCC TGT CCC</td>
</tr>
<tr>
<td>HIV-2 CD3R</td>
<td>TCG AGT TAA AGA CAG GAA CAG CTA TGT CGA</td>
</tr>
<tr>
<td>HTLV I-LTR</td>
<td>TCG AGG GGA GGA AAT GGG TGT CGA</td>
</tr>
<tr>
<td>PEA3</td>
<td>TCG AGC AGG AAG TGA CGT CGG</td>
</tr>
<tr>
<td>STROM</td>
<td>TCG AGC AGG AAG CAT TTC CTG GTC GC</td>
</tr>
<tr>
<td>p53-bax1</td>
<td>AGC TTT CTC ACA AGT TAG AGA CAA GCC TGG GCG TG GCT ATA TTG AAG CT</td>
</tr>
<tr>
<td>p53-reg</td>
<td>AGC TTT GCC TGG ACT TGC CTG CCC TTG CTT CTA AGC T</td>
</tr>
<tr>
<td>PAX-2</td>
<td>AGC ACC GTT CCG CTC AGC ACC GTT CCG CTC AGC ACC GTT CCG CTC</td>
</tr>
<tr>
<td>PAX-8</td>
<td>CTA AGC TTG AGT GGG CAT CAG AGC ATG GAG TC</td>
</tr>
<tr>
<td>YY1</td>
<td>TGC CTT GCA AAA TGG CGT TAC TGC AG</td>
</tr>
</tbody>
</table>

* underlined sequence refers to mutated nucleotides
**Preparation of Nuclear Lysates.** Nuclear lysates were collected as described previously (279). Confluent endothelial cells were harvested using trypsin-EDTA, washed twice with PBS at 4 °C and resuspended gently in 400 µl of Buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF) and incubated on ice for 15 min. To disrupt cytoplasmic membranes 25 µl of ice cold 10% NP40 was added and the mixture was gently vortexed. Nuclei were pelleted (10000g, 5 min.) at 4 °C and resuspended in ice cold Buffer C (20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF) and rocked vigorously for 15 min. at 4 °C. The mixture was subjected to centrifugation (10000g) for 5 min. at 4 °C and the supernatant was stored in aliquots at -80 °C. Protein concentration of the supernatant was determined using the Bradford method (as above). Passages 3 to 5 of BAEC were used for the EMSA assays.

**Electrophoretic Mobility Shift Assays (EMSA).** Single-stranded oligonucleotides were synthesized using the Beckman Oligo 1000 DNA synthesizer (Table III). Complementary strands were annealed as follows: 1000 pmol each, gel-purified sense and anti-sense oligonucleotides were mixed with polynucleotide kinase buffer (New England Biolabs Ltd., Mississauga, Ont.) to a total volume of 100 µl and the products analyzed on analytical agarose gels. The annealing mix was incubated at 80 °C for 5 minutes. Two pmol of the double-stranded oligonucleotide probes (Table III) were end-labeled with 60 µCi of ^32^P-γ-ATP using 1 µl of T₄ polynucleotide kinase (NEB) and 1 µl of 10X T₄ polynucleotide kinase buffer. The labeling mix was incubated at 37 °C for 90 minutes and then passed through a Sephadex G50 column (Bio-Rad Laboratories). The Sephadex column was prepared and then washed 3X with 100 µl of TEN buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 100 mM NaCl). To the probe reaction, 90 µl of TEN buffer and 2 µl of 10 mg/ml tRNA was added and the labeling mix was
applied to the G50 column. The column was then centrifuged at 1600g for 4 minutes. One μl of the labeled probe was diluted in 14 μl of CYTOSCIINT (ICN, Costa Mesa, CA) and then counted in a scintillation counter.

For EMSA reactions, 3-10 μg of BAEC nuclear extract were incubated for 10 minutes on ice in 20 μl of binding buffer (25 mM Tris-HCl (pH 8.0), 50 mM KCl, 6.25 mM MgCl₂, 10% glycerol, 50 μg/ml bovine serum albumin, 1 μg poly dIdC). Two fmol of labeled probe (2.5 X 10⁴ dpm) was added to the binding reaction mix and incubated at 22 °C for 50 minutes.

Where appropriate, non-labeled ('cold'), competitive oligonucleotides were added 10 minutes before addition of labeled probe. In reactions where recombinant Sp1 protein (Promega, Madison, WI) or recombinant YY1 (Santa Cruz) were used in EMSA, 0.03 % NP40 was added to the binding reaction and 0.05 % NP40 was added to the acrylamide gel. For "supershift" analyses, antibodies were added 30 minutes after probe addition and incubated a further 20 minutes at 22 °C. Binding reactions were size-fractionated on a non-denaturing, 4% acrylamide gel (37:1, mass:mass, acrylamide:NN’-methylenebisacrylamide), run at 200 Volts at 4 °C for 2 h in 0.5X Tris-Borate buffer. The gel was subsequently dried and autoradiographed at -80 °C with intensifying screens. Monoclonal and polyclonal antibodies were from Santa Cruz Biotechnology: Sp1 (PEP2), Sp2 (K-20), Sp3 (D-20), Sp4 (K-20), YY1 (H-414), GATA-2 (CG2-96), Ets-1 (NH₂-terminus. N-276), Ets-1 (COOH-terminus. C-20), Ets-2 (C-20), PU.1 (Spi-1, T-21), Erg-1 (C-17), Fli-1 (C-19), PEA3 (16) and Elk-1 (I-20). Anti-Elf-1 (rabbit polyclonal), anti-MAZ (murine monoclonal) and anti-GATA-2 (rabbit polyclonal) were generous gifts from J. M. Leiden (Chicago, IL) (179), K. B. Marcu (Stony Brook, NY) and S.H. Orkin (Boston, MA), respectively.
Transient Transfection Assays. All transient transfections were carried out as described in Chapter II. A CMV-based heterologous eukaryotic expression cassette for Elf-1 (pcDNA1.neo-Elf-1) was kindly provided by J. M. Leiden (Chicago, IL). For Drosophila Schneider studies, cells were co-transfected with 1 μg of experimental promoter/luciferase construct, the indicated amount of expression plasmids and 0.5 μg of a β-galactosidase construct driven by a Drosophila promoter, pADH-β-gal. The total amount of DNA transfected was kept constant (2 μg) with the addition of pPacUO. SL2 cells were seeded at a density of 2-3 X 10⁶ cells/ml at the time of transfection.

Protein Extraction. 48 hours after transfection, cells were harvested for protein extraction. BAEC culture was washed once with Hank’s Balanced Salt Solution without Mg²⁺ or Ca²⁺ (Gibco BRL) and then lysed with 300 μl of lysis buffer (0.1 M potassium phosphate buffer (pH 7.8), 1% Triton X-100, 1mM DTT, 2mM EDTA). Triton X-100 is a non-ionic detergent, which solubilizes the luciferase enzyme thus little enzyme remains bound to the cellular debris. The luciferase enzyme is most stable at a pH between 7.5 and 8.0 and thus a potassium buffer of pH 7.8 is used. 1 mM DTT is added to aid in the enzyme stability and 2 mM EDTA is added to chelate heavy metal ions which may interfere with luciferase activity. The lysis buffer was evenly distributed over the surface of the dish. The cell culture dishes now containing 300 μl of lysis buffer were incubated on the Belly Dancer apparatus (BIO/CAN Scientific, Mississauga, Ont.) for 20 minutes, at 4 °C. Following lysis, the cells were scraped and collected into Eppendorf tubes. Protein extracts were centrifuged at 14000 rpm in a Beckman model centrifuge for 2 minutes to pellet residual cellular debris. The supernatants were then transferred to a fresh
tube where they were either immediately assayed for luciferase activity or stored at -80 °C for subsequent assays that would assess β-galactosidase activity and protein concentration.

**Luciferase Assays.** Cell extracts were assayed for luciferase activity immediately after harvesting. Luciferase activity was measured using the MonoLight 2010C luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI).

To assay for luciferase activity 25 µl of protein extract is used. The synthetic substrate used for this assay is D-Luciferin (D-(-)-2-(6'-hydroxy-2'-benzothiasolyl)-thiazoline-4-carboxylic acid). 100 µl of D-Luciferin (1 mM) and 100 µl of luciferase assay buffer (30 nM Tricine (Calbiochem, La Jolla, CA), 3mM ATP, 15 mM MgSO₄, 10mM DTT, 1mM Coenzyme A (Calbiochem, La Jolla, CA), pH 7.8) are automatically injected by the dual injection system of the luminometer. Coenzyme A is a compound used to sustain light output for an extended period of time (4). By affecting the luciferase kinetics, Coenzyme A reacts with the inhibitory oxyluciferin-enzyme complex to form free enzyme, AMP and oxyluciferyl-CoA. This ultimately results in the removal of the inhibitory complex and a faster turnover rate of enzyme. Measurements of light units were integrated over a 10 second period.

Data in raw luciferase units (RLU) is normalized for the non-specific background of mock-transfected cells, which represented = 0.5% of most experimental luciferase activities. Intra- and inter-assay coefficients of variation averaged 7% and 9%, respectively.

**β-Galactosidase Assay.** β-D-galactosidase is encoded for by the *LacZ* gene of E. Coli. For efficient translation in eukaryotic cells the translation initiation site has been optimized. This
activity is used as a measure of transfection efficiency from dish to dish. CPRG (chlorophenol red-β-D-galactopyranoside, C₂₅H₂₁O₁₀Cl₂SNa) is the chromogenic substrate used for β-D-galactosidase. 40 µl of cell extract was mixed with 139 µl of 0.1 M sodium phosphate buffer (pH 7.3), 3.6 µl of 0.5 M MgCl₂, 16 µl of 0.1 M CPRG solution, and 1.4 µl of 14.3 M 2-mercaptoethanol. The final volume of the reaction mix was 200 µl. CPRG was dissolved in 0.1 M sodium phosphate buffer (pH 7.3), made fresh before each assay. The reaction mix was thoroughly vortexed, spun down and incubated at 37 °C for 20 minutes. A bright, ruby red colour appeared by the end of the incubation period. To stop the reactions, 500 µl of 1 M Na₂CO₃ is added to each tube. 1 M Na₂CO₃ was made fresh before each assay and it was used to adjust the pH of the reaction to approximately 11, stopping further enzymatic conversion of CPRG. The reaction mixtures were thoroughly mixed, spun down and transferred to disposable cuvettes (SARSTEDT, St. Laurent, Quebec) and immediately read for optical density at 570 nm.

Control reactions used in this assay include a positive and a negative control. As a negative control, 40 µl of mock-transfected cell extract was used. As a positive control, 40 µl of mock-transfected cell extract was used along with 1 µl of β-galactosidase (Sigma, 50 Units/ml). The negative control will give a measure of the endogenous β-galactosidase activity and at the end of the incubation period the reaction mix is usually yellow in colour, whereas the positive control is bright, ruby red.

The β-galactosidase activity of the experimental dishes was corrected for background. This calculation permits the measurement of the β-galactosidase activity contributed by the transfected constructs themselves. The product of the calculation was then used in the normalization of the data.
**Measurement of Protein Concentration.** A Bio-Rad Protein Assay (Bradford) was performed to determine the concentration of solubilized protein in each dish. This procedure is a dye-binding assay in which a differential colour change of a dye occurs in response to various concentrations of protein. 25 µl of protein extract was diluted 10-fold in 225 µl of distilled water. The Bradford Reagent (Bio-Rad) was diluted 5-fold in distilled water and filtered through a 0.22 µm filter using a syringe. 2 ml of this acidic dye was then added to 40 µl of diluted cell extract, mixed by inversion, transferred to a disposable cuvette (SARSTEDT, St. Laurent, Quebec) and subsequently measured at an optical density of 595 nm with a Pharmacia LKB Ultrospec Plus spectrophotometer after a 5 minute incubation period. The absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 dye shifts from 465 nm to 595 nm when protein binding occurs. Coomassie blue dye binds primarily basic and aromatic amino acid residues, especially arginine.

The measurements are ultimately compared to a standard curve created with bovine serum albumin to provide a relative measurement of protein concentration. This assay is used to account for protein variability from dish to dish in each transfection experiment.

**DNA Analysis.** It is essential to accurately control for the amount and quality of transfected DNA used per dish given that studies are performed under conditions in which luciferase activity is directly proportional to the amount of transfected DNA. The concentration of maxi-preparative DNA was accurately determined using three methods. A DyNA Quant 200 Fluorometer (Hoefer Pharmacia Biotech Inc.), an Ultrospec Plus UV/Visible Spectrophotometer
(Pharmacia Biotech, Uppsala, Sweden) and uncut DNA subjected to gel electrophoresis were used to accurately quantitate the DNA.

**Data Analysis.** Unless otherwise indicated, data are expressed as the mean ± S.E.M. obtained in at least three independent transfection experiments, each done in triplicate. Comparisons were made with analysis of variance (ANOVA), followed by the Student-Newman-Keuls test. The level of statistically significant difference was defined as p < 0.05.
III.4 RESULTS

III.4.1 TRANS-ACTING FACTOR INTERACTION STUDIES USING EMSA ANALYSIS

III.4.1.1 Nucleoprotein Complexes Formed by PRD 1

A 30-mer double-stranded DNA probe (-120/-91) representing residues -120/-91 in the human eNOS 5'-flanking region, and thereby spanning PRD 1, formed a series of protein/DNA complexes with BAEC nuclear extracts (Figure 16A. complexes A. B. C and D. lane 2). Similar protein/DNA complexes were observed with HUVEC nuclear extracts (Figure 16C). With the addition of 50-fold and 100-fold molar excess of unlabeled -120/-91 probe all 4 protein/DNA complexes were effectively competed away (Figure 16C. lanes 4 and 5). With the addition of 100-fold molar excess of an oligonucleotide containing a consensus Sp1 site, all 4 complexes (A. B. C and D) were competed away (Figure 16A. lane 3). Unlabeled oligonucleotides with a mutation in the Sp1 binding site (\(^5\)'-GGGcGtC-3') located at -104 were ineffectual in competition studies, whereas mutating the GATA site (\(^5\)'-aATA-3') at -108/-105 had no effect on competition (Figure 16A. lanes 4 and 5, respectively). When protein/DNA complexes were incubated with a monoclonal antibody directed against Sp1 a "supershift" of complex A was evident (Figure 16B. lane 4 and 15C. lane 6). Using monoclonal and polyclonal antibodies directed against GATA-2, no supershift or shift abrogation was observed. For instance, minor bands evident in Figure 16A. lane 5 were not affected by anti-GATA (data not shown). Incubating protein/DNA complexes with polyclonal antibodies directed against Sp2 (data not shown) and Sp4 (Figure 16B. lane 2), resulted in no supershift or shift abrogation. However.
Figure 16. EMSAs of endothelial nuclear protein binding to -120/-91 PRD I eNOS 5'-flanking region. (A) Lane 1 represents probe alone and in lane 2, 3 μg of BAEC nuclear extract was added. Four specific protein/DNA complexes are evident: A, B, C and D. In lanes 3-5, 100-fold molar excess of Sp1 consensus, -120/-91mutSp1 and -120/-91mutGATA oligonucleotides were added to the binding reaction as competitors. (B) Lane 1 represents -120/-91 probe incubated with 3 μg of BAEC nuclear extract. In lanes 2-4, anti-Sp4, -Sp3 and -Sp1 were added to the binding reaction, respectively. 2.5 X 10^4 dpm of labeled -120/-91 probe was used in each binding reaction. (C) Lane 1 represents probe alone, lanes 2 and 3 represent 5 μg and 3 μg, respectively of HUVEC nuclear extract incubated with probe. In lanes 3 and 4, 50- and 100-fold molar excess of unlabelled -120/-91 probe was added. An antibody directed against Sp1 was added in lane 5. (D) Lane 1, -120/-91 probe alone: lane 2, -120/-91 probe, Sp1 protein: lane 3, -120/-91 probe, Sp1 protein, anti-Sp1. (E) Competition studies showing the specificity of the Sp1/-120/-91 probe complex. Lane 1 & 7, -120/-91 probe alone: lane 2 & 8, -120/-91, Sp1 protein: lane 3-6, -120/-91 probe, Sp1 protein and 50-fold, 100-fold, 500-fold, 1000-fold molar excess unlabelled -120/-91 competitor respectively; lanes 9-12, same as lanes 3-6 except competitor used was unlabelled heterologous consensus Sp1 sequence. 2.5 X 10^4 dpm of labeled -120/-91 probe was used.
exposing protein/DNA complexes to a polyclonal antibody directed against Sp3 resulted in both supershift and shift abrogation of complexes B and C (Figure 16B, lane 3). A specific protein/DNA complex formed when the -120/-91 probe was utilized in an EMSA with Sp1 but not AP-2 and was supershifted with an antibody directed against human Sp1 (data not shown). Competition studies performed with unlabeled -120/-91 probe and a heterologous consensus Sp1 high-affinity site from the early SV-40 promoter confirmed the specificity of this reaction (Figure 16D and E).

III.4.1.2 Nucleoprotein Complexes Formed by PRD II

A series of protein/DNA EMSA complexes were observed with the labeled -140/-111 probe and BAEC nuclear extracts (Figure 17A, lanes 2 and 3). Adding 100-fold molar excess of unlabeled -140/-111 probe resulted in the disappearance of these complexes, suggesting that they are specific (Figure 17A, lane 4). Competition was also observed with the addition of 100-fold molar excess of unlabeled -155/-120 (Figure 17B, lane 4) (Table III). Sequence inspection of PRD II regions indicated a variety of putative DNA cis-elements: Ets [GGAA/T] (168), YY1 [5'-CCATT-3'] (91, 118), low affinity Sp1 [5'-GGGAGG-3'] (14), MAZ [GGGAGGG] (33, 74), p53 half-site [5'-GGGCTTGTTCT-3'] (323), and paired domain PAX family members [5'-GTTC-3'] (44). Competitor oligonucleotides containing consensus Pax-2, Pax-8 or a variety of p53 binding sites had no effect on the formation of nucleoprotein complexes (data not shown). Multiple consensus p53 recognition sites were used given that neighbouring DNA sequences and the phosphorylation state of p53 influence binding site selection. In contrast to these negative findings, unlabeled oligonucleotides containing various Ets-family member binding sites were very effective in competition studies (Figure 17A). For example, the long terminal repeat sequence of
Figure 17. EMSAs of BAEC nuclear protein binding to -140/-111 PRD II eNOS 5'-flanking region. (A) Lane 1 represents probe alone. Lanes 2-3 represent the addition of 3 and 10 μg of BAEC nuclear extract, respectively. In lanes 4-8, 250-fold molar excess of various competitor oligonucleotides were added to 3 μg of BAEC nuclear extract: unlabelled -140/-111 (lane 4), HIV-2 CD3R (lane 5), HTLV I-LTR (lane 6), STROM (lane 7) and PEA3 (lane 8) (2.5 X 10^4 dpm of labeled probe). (B) Lane 1 represents -140/-111 probe alone and 10 μg of BAEC nuclear extract was added in lane 2. 100-fold molar excess of unlabelled -140/-111 and -155/-120 was added in lanes 3 and 4. An antibody directed against Elf-1 was added in lane 5. (C) Lane 1 represents probe alone and 10 μg of BAEC extract was added in lane 2. 100-fold molar excess of cold -140/-111 was added in lane 3. An antibody directed against MAZ was added in lane 4. (D) Lane 2 represents probe alone and 10 μg of BAEC extract was added in lane 1. 100-fold molar excess of cold -140/-111 and YY1 oligonucleotides was added in lanes 3 and 4, respectively. (E) EMSAs were performed with an HIV-2 CD3R LTR probe containing an Elf-1 recognition sequence and BAEC nuclear extracts. Lane 1 represents probe alone (2.5 X 10^4 dpm labeled probe) and 10 μg of BAEC nuclear extract was added in lane 2. 100-fold molar excess of unlabelled competitor DNA was added in lane 3 (HIV-2 CD3R), lane 4 (-155/-120), lane 5 (-140/-111) and lane 6 (-120/-91). Anti-Elf-1 was added in lane 7. For all gels, arrows on the left represent protein/DNA complexes and arrows on the right represent supershifted complexes and/or abrogated complexes.
the HIV-2 promoter has been reported to bind Elf-1, an Ets family member (147) and as shown in Figure 17A (lane 5), demonstrated clear competition. Other Ets binding site-containing oligonucleotides, including the human T-cell lymphotrophic virus type I long terminal repeat (HTLV-I LTR) site (Ets-1) (106), the rat stromelysin 1 promoter site (Ets-2) (331) and the polyomavirus PEA3 site (PEA3) (330) also competed, though to a lesser extent (Figure 17A, lanes 6 to 8). Addition of monoclonal or polyclonal antibodies directed against varied members of the Ets family (Ets-1, Ets-2, PU.1, Erg-1, Fli-1, PEA3 and Elk-1) failed to modify nucleoprotein complexes formed with the -140/-111 probe. These antibodies have been demonstrated to exhibit cross-reactivity across species. However, our findings are complicated by the well described difficulties inherent in characterizing which specific members of the Ets family are implicated in the formation of protein/DNA complexes in nuclear extracts and may be related, in part, to the contribution of the autoinhibitory binding domain in Ets family members (151, 249, 294). Exposure of complexes formed with the -140/-111 probe to a rabbit polyclonal antibody directed against Elf-1 resulted in a supershift of the fastest migrating complex (Figure 17B, lane 5). These findings imply that the fast migrating complex contains a protein that is antigenically-related to Elf-1. To confirm that an Elf-1-like protein is present in the BAEC nuclear extracts, the HIV-2 LTR oligonucleotide probe was labeled and incubated with BAEC nuclear extract (Figure 17E). This activator recognition sequence is known to bind Elf-1 in T lymphocytes (179). Addition of 100-fold molar excess of -140/-111 and -155/-120 probes resulted in competition (Figure 17E, lanes 4 and 5) of the fast migrating protein/DNA complex and anti-Elf-1 resulted in a supershift of the fastest migrating complex (Figure 17E, lane 7). These findings are taken to indicate that Elf-1 is present in endothelial cell nuclear extracts and that this Ets family member can participate in nucleoprotein complex formation with PRD II.
YY1 is a C2H2-type zinc finger DNA-binding protein known to bind to consensus DNA sequences evident in PRD II [5' -CCATT- 3'] (91, 118). To test whether YY1 protein bound to PRD II, the -140/-111 probe was exposed to a competitor oligonucleotide containing the YY1 site found in the upstream conserved region of Moloney murine leukemia virus (91) (Figure 17D, lane 4). A clear reduction in one of the fastest migrating bands is evident upon addition of this competitor, suggesting that YY1 participates in the formation of protein/DNA complexes formed upon PRD II. Recombinant YY1 protein also formed protein/DNA complexes with the -140/-111 PRD II probe in EMSA (data not shown).

A second PRD II probe corresponding to eNOS promoter sequence spanning -155 to -120 also formed a series of unique and specific protein/DNA complexes (Figure 18A, lane 2 and 3). Addition of 100-fold molar excess of cold -140/-111 resulted in competition for some, but not all of the complexes (Figure 18A, lane 4). This may suggest that the protein/DNA complexes that were not effectively competed with the -140/-111 oligonucleotide, represent interactions of trans-acting factors with portions of the -155/-120 probe that are not present on -140/-111. The slowest migrating complex disappeared with the addition of anti-Sp1 whereas the next two complexes failed to form upon addition of anti-Sp3 (Figure 18B, lanes 2 and 3, respectively). No change in complex pattern was observed with the addition of anti-Sp4 or anti-Sp2 (data not shown). Upon the addition of 100-fold molar excess of cold -120/-91, which contains a high-affinity Sp1 cis-DNA sequence, clear competition of these slow migrating complexes was evident (data not shown). In contrast, when 100-fold molar excess of cold -155/-120 was added as a cold competitor to the complexes formed with the -120/-91 probe, only slight competition was observed. Taken together, these data suggest the presence of a low-affinity Sp1 cis-DNA element on the -155/-120 probe, but not on the -140/-111 probe. When a polyclonal antibody
Figure 18. EMSAs of BAEC nuclear protein binding to -155/-120 PRD II eNOS 5' flanking region. (A) Lane 1 represents probe alone and 10 μg of BAEC nuclear extract was added in lane 2. 100-fold molar excess of unlabelled -155/-120 and -140/-111 probes were added in lanes 3 and 4, respectively (2.5 X 10^4 dpm of labeled probe). An antibody directed against Elf-1 was added in lane 5. (B) Lane 1 represents probe alone and 10 μg of BAEC nuclear extract was added in lane 2. Antibodies were introduced in lanes 3-5: anti-Spl (lane 3), -Sp3 (lane 4) and -MAZ (lane 5). For all gels, arrows on the left represent protein/DNA complexes and arrows on the right represent supershifted complexes and/or abrogated complexes.
directed against Elf-1 was added to the -155/-120 binding reaction a reduction was evident in the intensity of the fastest migrating specific complex and a supershift was seen (Figure 18A, lane 5).

MAZ (myc-associated zinc finger) is a zinc-finger transcription factor that displays protean roles in transcription initiation, interference and termination (33). MAZ was originally identified in the c-myc P2 promoter and is known, through binding site selection assays, to bind to [GGGAGGG]- or [CCCTCCC] (CT elements) (33, 74). Putative MAZ-binding CT elements were identified in the eNOS promoter at -191, -146, -99, -75, -62, and -47. An important facet of MAZ function is the participation of partner proteins. For instance, enhancer activity of the CD4 gene is critically dependent upon MAZ and an Ets consensus site that binds Elf-1 (80, 343). Functional interactions between MAZ and Sp1 occur with a number of genes, including the adenovirus major late and the TATA-less serotonin 1a receptor promoters (243). Because proteins shown to functionally interact with Ets and/or Sp1 family members in other promoters became candidate participants for nucleoprotein complex formation upon PRD II, studies assessed MAZ binding. A monoclonal antibody directed against MAZ resulted in shift abrogation of complexes formed upon the -155/-120 probe, especially one of the prominent fastest migrating ones (Figure 18B, lane 5). Consistent with protein-protein interactions involving MAZ with Sp1 and/or Sp3, a clear reduction in the slower migrating protein/DNA complexes was also observed following the addition of MAZ antibody. Protein/DNA complexes formed by the -140/-111 probe also demonstrated a clear reduction with the addition of MAZ antibody (Figure 17C, lane 4).

In summary, studies of endothelial cell nuclear extracts and double-stranded oligonucleotide probes representing PRD I and PRD II functional domains demonstrate nucleoprotein complexes
composed of Sp1, variants of Sp3, Ets-1, Elf-1, MAZ and YY1. The majority of protein/DNA complexes seen at this EMSA resolution have been accounted for.

III.4.2 TRANSIENT TRANSFECTION FUNCTIONAL STUDIES IN DROSOPHILA SCHNEIDER SL2 CELLS

III.4.2.1 Sp1 and Ets Family Members are Essential Activating Components of PRD I and PRD II

Functional promoter analyses in endothelial cells revealed that mutating activator regions encompassing high- and low-affinity Sp1/Sp3 recognition sequences (-104/-95 and -146/-141) resulted in a reduction of eNOS promoter/reporter activity (Figure 11 and 13). Similarly, mutating the region encompassing an Ets recognition sequence (-129/-126) also resulted in a reduction of promoter activity (Figure 11 and 13). Based upon this background, a model can be proposed wherein Sp1, Sp3 and Ets family members are essential for in vivo eNOS promoter function. To evaluate this hypothesis, a series of transient transfection experiments were performed in cells which lack constitutive Sp1, Sp3 and Ets activities, namely the Drosophila Schneider cell line (for each experimental series n = 3, triplicate determinations) (63, 117). Ets-1 is a well-characterized member of the Ets family of transcription factors that is known to be robustly expressed in endothelial cells (335, 336). In the absence of Sp1, Sp3 or Ets-1 the pGL2-1193/+109 reporter construct exhibited trivial functional activity, being essentially equivalent to mock-transfected cells. As shown in Figure 19, cotransfection of increasing amounts of Sp1 expression cassette, over the range 1 to 250 ng/plate, resulted in a concentration-dependent increase in functional promoter activity (n=3, triplicate determinations). 250 ng of Sp1
Figure 19. Sp1 and Sp3 transactivate human eNOS promoter/reporter luciferase constructs in Drosophila Schneider cells. Assay of promoter activity of pGL2-1193/+109 promoter/reporter luciferase construct upon co-transfection with increasing amounts of pPacUSp1 (5 to 250 ng, left panel), pPacUSp3 (5 to 250 ng, middle panel) and pPacUSp3 (5 to 250 ng) with half-maximal amounts of pPacUSp1 (40 ng) (left panel). Shown are representative experiments (triplicate determinations), each performed 4 times. Data are expressed as fold increase in luciferase activity ± S.E.M. relative to pGL2-1193/+109.
expression plasmid resulted in a maximal 140-fold increase. Although, transfecting increasing amounts of Sp1 expression cassette correlated with increases in eNOS promoter activity, we did not directly demonstrate that more Sp1 protein was made with the addition of more DNA. Thus, a caveat in our interpretations must be added. To address this, Western blot analysis could be performed on protein extracts isolated from cells which have been previously transfected with increasing amounts of Sp1 expression plasmid. We anticipate that this will demonstrate an increase in Sp1 production as reported by others (216). Similarly, EMSA analysis using a consensus Sp1 oligonucleotide may be performed to demonstrate increased protein-DNA complex formation with increasing amounts of transfected expression plasmid.

 Increasing amounts of expression cassette encoding full-length Sp3, over the range 1 to 250 ng/plate, also stimulated luciferase activity in a concentration-dependent fashion, with a maximal 35-fold increase (Figure 19). Co-expression of Sp3 with half-maximal amounts of Sp1 (15-40 ng) resulted in increased functional promoter activity of the eNOS promoter compared with either Sp1 or Sp3 alone (Figure 19). Transfecting increasing amounts of Ets-1 expression plasmid alone failed to modify functional eNOS promoter activity over the range 1 to 250 ng/dish (Figure 20). However, co-expression of Ets-1 with half-maximal amounts of Sp1 resulted in a positive functional interaction with the eNOS promoter (Figure 20) (n=3, triplicate determinations).

To determine whether multiprotein complexes are involved in the cooperative control of constitutive expression of the human eNOS gene various Sp1 family members and Ets proteins were cotransfected alone or in combination with each other. Though threshold amounts of Ets-1, Sp3 and Sp1 expression cassettes (5 ng) alone had minimal effects on the pGL2-1193/+109
Figure 20. Sp1 and Ets-1 transactivate human eNOS promoter/reporter luciferase constructs in Drosophila Schneider cells. Assay of promoter activity of pGL2-1193/+109 promoter/reporter luciferase construct upon co-transfection with increasing amounts of pPacUSp1 (5 to 250 ng, left panel), pPacUEts-1 (5 to 250 ng, middle panel) and pPacUEts-1 (5 to 250 ng) with half-maximal amounts of pPacUSp1 (40 ng)(left panel). Shown are representative experiments (triplicate determinations), each performed 4 times. Data are expressed as fold increase in luciferase activity ± S.E.M. relative to pGL2-1193/+109.
Figure 21. Sp1, Sp3 and Ets-1 transactivate human eNOS promoter/reporter luciferase constructs in Drosophila Schneider cells. Assay of promoter activity of wild-type and linker-scanning mutant pGL2-1193/+109 promoter/reporter luciferase constructs following co-transfection with threshold amounts of pPacUSp1 (5 ng), pPacUSp3 (5 ng) and/or pPacUEts-1 (5 ng). Shown are representative experiments (triplicate determinations), each performed 4 times. Data are expressed as fold increase in luciferase activity ± S.E.M. relative to pGL2-1193/+109.
luciferase reporter construct (Figure 21) (n=3, triplicate determinations), the combined addition of threshold amounts of Ets-1, Sp3 and Sp1 activated the eNOS promoter in a cooperative fashion. For example, the addition of 5 ng of Ets-1 expression plasmid resulted in a 5-fold increase in functional promoter activity compared to the combined addition of Sp1 and Sp3 alone. These results highlight the cooperative and complex nature of Ets-1, Sp3 and Sp1 interactions in functional eNOS promoter activity and underscore their essential contributions to nucleoprotein complex formation for the eNOS promoter. To demonstrate that these factors were acting through cis-DNA sequences found in PRD I and II, these factors were co-transfected with 3 linker-scanning mutant constructs. Co-transfection of the PRD I linker-scanning mutant construct with threshold amounts of Sp1, Sp3 and Ets-1 demonstrated an approximate 80% decrease in activity relative to the activity of the wild type eNOS construct (Figure 21). When activator sequences between -144 and -135 in PRD II were mutated an approximate 50% decrease in functional promoter activity was observed confirming the important contribution of 5’-regions of PRD II. When sequences corresponding to the Ets recognition site in PRD II were mutated, an approximate 70% decrease in functional promoter activity was observed (Figure 20). These results identify PRD I and II as critical activator recognition sequences for eNOS promoter function. Mutating the Sp1/Sp3 sites in PRD I and II and mutating the Ets site in PRD II resulted in dramatic decreases in functional promoter activity both in endothelial cells and the Drosophila Schneider heterologous expression system.
MAZ can both activate and inhibit transcription initiation (33). The functional contribution of MAZ to eNOS promoter/reporter was assessed in Drosophila Schneider cells activity using co-transfection experiments. Cotransfection of increasing amounts of MAZ expression plasmid alone, over the range 1 to 250 ng/plate, did not have any effect on activity of the -1193/+109 eNOS promoter/reporter luciferase construct (Figure 22) (n=3, triplicate determinations). Addition of 100 ng of MAZ expression construct to threshold amounts of Ets-1, Sp3 and Sp1 resulted in an approximate 90% decrease in functional promoter activity relative to the addition of threshold amounts of Ets-1/Sp3/Sp1 alone (Figure 23) (n=3, triplicate determinations). This suggested that MAZ has a negative effect on the cooperative interaction between Ets-1, Sp3 and Sp1. MAZ had a similar effect on Sp1 alone (Figure 24) (n=3, triplicate determinations). An approximate 95% drop in activity was observed when 100 ng of MAZ expression construct was added to a half-maximal dose of Sp1, compared to Sp1 alone. Various linker-scanning mutant constructs were used to define the requirements for PRD I and PRD II cis-elements (Figure 25) (n=3, triplicate determinations). A lower amount of MAZ (5 ng) resulted in a 60% reduction of wild type functional promoter activity relative to Ets-1/Sp3/Sp1 alone. Though baseline activity was obviously lower. MAZ was still able to repress promoter/reporter activity directed by PRD I (-104 / -95) or PRD II (-144/-135) mutants. In contrast, MAZ failed to inhibit functional activity when -134/-125 regions of PRD II was mutated (-134/-125 mut). This suggested that the Ets site located in PRD II is necessary for MAZ to exhibit its maximal repressive effect. In summary, these results suggest (i) that MAZ exhibits a negative effect on eNOS promoter activity in Drosophila Schneider cells, and (ii) that this repressor activity is especially dependent on the Ets site in PRD II.
Figure 22. Effect of MAZ on eNOS promoter activity in *Drosophila* Schneider cells. Promoter activity of pGL2-1193/+109 construct following co-transfection with increasing amounts of pPacUMAZ (5 to 250 ng). Shown are representative experiments (triplicate determinations), each performed 3 times. Data are expressed as fold increase in luciferase activity ± S.E.M. relative to pGL2-1193/+109.
Figure 23. Effect of MAZ on Sp1/Sp3/ets-1-mediated eNOS promoter activity in *Drosophila Schneider* cells. MAZ inhibits pGL2-1193/+109 promoter/reporter luciferase construct activity in the presence of threshold amounts of pPacUSp1 (5 ng), pPacUSp3 (5 ng) and pPacUEts-1 (5 ng). Shown are representative experiments (triplicate determinations), each performed 3 times. Data are expressed as fold increase in luciferase activity ± S.E.M. relative to pGL2-1193/+109.
Figure 24. Effect of MAZ on Sp1-mediated eNOS promoter activity in Drosophila Schneider cells. MAZ (pPacUMAZ, 100 ng) inhibits pGL2-1193/+109 promoter/reporter luciferase construct activity in the presence of half-maximal amounts of pPacUSp1 (15 ng). Shown are representative experiments (triplicate determinations), each performed 3 times. Data are expressed as fold increase in luciferase activity ± S.E.M. relative to pGL2-1193/+109.
Figure 25. Effect of MAZ on eNOS promoter activity in Drosophila Schneider cells using linker-scanning mutant constructs. Effect of MAZ (pPacUMAZ, 5 ng) on promoter activity of wild-type and linker-scanning mutant pGL2-1193/+109 promoter/reporter luciferase constructs following co-transfection with threshold amounts of pPacUSp1 (5 ng), pPacUSp3 (5 ng) and pPacUEts-1 (5 ng). Shown are representative experiments (triplicate determinations), each performed 3 times. Data are expressed as fold increase in luciferase activity ± S.E.M. relative to pGL2-1193/+109.
In mammalian cells the nucleoprotein Sp3 exists as 3 protein isoforms as a result of alternate usage of translational initiation sites (160). One isoform with an apparent molecular weight of 110 kDa represents the full-length Sp3 protein, whereas the other two isoforms with apparent molecular weights of 80 and 78 kDa represent internal AUG-initiated variants of Sp3. These smaller Sp3 variants, which lack the NH2-terminal transactivation domain, are still capable of binding Sp1 recognition elements but may have a repressive effect on transcription depending upon the number of binding sites within the cellular promoter (160). The molecular characterization of these Sp3 variants presumably accounts for the faster migrating DNA/complexes evident in a variety of mammalian cell lines using EMSA (160). Given the above findings that EMSA using endothelial nuclear extracts demonstrate protein/DNA complexes consistent with these Sp3 variants we sought to evaluate the functional consequences of removing the Sp3 trans-activation domain on eNOS promoter function. As shown in Figure 26A (n=3, triplicate determinations), NH2-terminal deleted Sp3 expression cassette (Sp3ΔNH2) was no longer capable of trans-activating the promoter by itself over the range 1 to 250 ng/plate (compare to Sp3 in Figure 19). Sp3ΔNH2, however, dramatically enhanced the cooperative activity exhibited by threshold amounts of Ets-1, Sp3 and Sp1. at both 5 and 100 ng of Sp3ΔNH2 (Figure 26B) (n=3, triplicate determinations). On the other hand, when varied amounts of Sp3ΔNH2 were co-transfected with half-maximal amounts of Sp1 expression construct (15-40 ng), a biphasic effect on Sp1-mediated activation of the eNOS promoter was observed (Figure 26C) (n=3, triplicate determinations). A low amount of Sp3ΔNH2 (1 ng) exhibited a repressive effect on Sp1-mediated activation, whereas cooperativity was demonstrated between Sp1 and higher amounts of Sp3ΔNH2 (250 ng). Low amounts of Sp3ΔNH2 was also able to repress full-
Figure 26. Effect of NH2-deleted Sp3 (Sp3ΔNH2) on eNOS promoter activity in Drosophila Schneider cells. (A) Promoter activity of pGL2-1193+/+109 construct following co-transfection with increasing amounts of pPacUSp3(-Nterm) (1 to 250 ng). (B) Sp3_NH2 (pPacUSp3(-Nterm), 5 and 100 ng) augments pGL2-1193+/+109 promoter/reporter luciferase construct activity in the presence of threshold amounts of pPacUSp1 (5 ng), pPacUSp3 (5 ng) and pPacUEts-1 (5 ng). (C) Effect of low (1 ng) and high (100 ng) concentrations of Sp3_NH2 (pPacUSp3(-Nterm)) on Sp1-induced (pPacUSp1, 15 ng) activation of eNOS promoter activity. Shown are representative experiments (triplicate determinations), each performed 3 times. Data are expressed as fold increase in luciferase activity ± S.E.M. relative to pGL2-1193+/+109.
length Sp3-mediated activation (100 ng) of the eNOS promoter in Drosophila Schneider cells (data not shown).

### III.4.2.4 Functional Studies Evaluating the Effect of Elf-1 on eNOS Promoter Activity in Drosophila Schneider and Endothelial Cells

Given the demonstration that Elf-1 accounts, in part, for endothelial PRD II nucleoprotein complexes we determined the functional properties of Elf-1 in Drosophila Schneider co-transfection experiments. Increasing amounts of Elf-1 expression construct alone, over the range 1 to 250 ng/plate, minimally enhanced eNOS promoter activity (Figure 27) (n=3, triplicate determinations). Surprisingly, co-transfection of Elf-1 elicited a concentration-dependent repressive effect on the functional activity of half-maximal amounts of Sp1 (15-40 ng) (Figure 28) (n=3, triplicate determinations). In other words, Elf-1 exerted a negative effect on the ability of Sp1 protein to activate the eNOS promoter in SL2 cells. In contrast, Elf-1 failed to repress eNOS promoter activity in the presence of Ets-1. Even 100 ng of Elf-1 expression cassette failed to inhibit the cooperativity evident when the -1193/+109 eNOS promoter/reporter construct was co-transfected with threshold amounts of Ets-1/Sp3/Spl expression plasmids (Figure 29) (n=3, triplicate determinations). Conversely, CMV-directed expression of Elf-1 protein in BAEC had a stimulatory effect on the -1193/+109 eNOS promoter/reporter construct (Figure 30). This augmentation of eNOS promoter activity occurred over a range of added Elf-1 heterologous eukaryotic expression cassette (100 ng to 1 μg). Elf-1 enhanced eNOS promoter activity 3.2 to 13.6 fold above expression vector alone (n = 4, triplicate determinations, 1 μg). Moreover, Elf-1-induced activation of functional promoter activity in BAEC was significantly blunted when Elf-1
was co-expressed with the -134/-125mut linker-scanning construct compared to the wild type -1193/+109 eNOS promoter/reporter construct (data not shown).
Figure 27. Effect of Elf-1 on eNOS promoter activity in Drosophila Schneider cells. Promoter activity of pGL2-1193/+109 construct in Drosophila Schneider cells following co-transfection with increasing amounts of pPacUElf-1 (15 to 250 ng). This is a representative experiment (triplicate determination), performed 3 times. Data are expressed as fold increase in luciferase activity ± S.E.M. relative to pGL2-1193/+109.
Figure 28. Effect of Elf-1 on Sp1-mediated eNOS promoter activity in *Drosophila* Schneider cells. Elf-1 inhibits pGL2-1193/+109 promoter/reporter luciferase construct activity in *Drosophila* Schneider cells in the presence of half-maximal amounts of pPacUSp1 (40 ng). This is a representative experiment (triplicate determination), performed 3 times. Data are expressed as fold increase in luciferase activity ± S.E.M. relative to pGL2-1193/+109.
Figure 29. Effect of Elf-1 on Sp1/Sp3/Ets-1-mediated eNOS promoter activity in Drosophila Schneider cells. Effect of Elf-1 (pPacUElf-1, 100 ng) on pGL2-1193/+109 promoter/reporter luciferase construct activity in Drosophila Schneider cells in the presence of threshold amounts of pPacUSp1 (5 ng), pPacUSp3 (5 ng) and pPacUEts-1 (5 ng). This is a representative experiment (triplicate determination), performed 3 times. Data are expressed as fold increase in luciferase activity ± S.E.M. relative to pGL2-1193/+109.
Figure 30. Effect of Elf-1 on eNOS promoter activity in BAEC. Elf-1 augments pGL2-1193/+109 promoter/reporter luciferase construct activity in BAEC (pcDNA1/neo-Elf-1, 1 μg). Data are expressed as fold increase in luciferase activity ± S.E.M. relative to pGL2-1193/+109. Shown are results from four experiments (triplicate determinations) expressed as fold increase in luciferase activity ± S.E.M. relative to empty expression vector alone.
III.5 DISCUSSION

Two tightly clustered activator regions were identified in proximal regions of the human eNOS promoter using deletion analysis and linker-scanning mutagenesis: positive regulatory domain I (PRD I) (-104/-95) and PRD II (-144/-115) (see Chapter II). Analysis of trans-factor binding and functional expression studies revealed a surprising degree of cooperativity and complexity in PRD I and PRD II structure and function. Through analysis of nucleoprotein complexes in endothelial cells and functional domain studies in Drosophila Schneider cells and endothelial cells we demonstrate positive and negative protein-protein cooperativity involving Sp1. variants of Sp3, Ets-1, Elf-1, MAZ and YY1. PRD I and II function is conserved across species given that the activity of human eNOS promoter/reporter constructs exhibited similar trends in activity in BAEC and HUVEC (Figure 8. 9 and 10). As well, there is a high degree of relatedness in human and bovine genomic DNA sequences for PRD I and PRD II (320) and no common allelic variants in these genomic regions for human eNOS have been detected (-475 to +152) (156).

Sp1, Sp2, Sp3 and Sp4 are closely related members of a gene family encoding zinc-finger (His2Cys2) transcription factors. Sp1, Sp2 and Sp3 are ubiquitously expressed, whereas Sp4 protein is primarily expressed in certain cell types of the brain. All four proteins contain a highly conserved DNA binding zinc-finger domain close to the COOH-terminus, and two glutamine- and serine/threonine-rich domains near the NH2-terminus. The latter domains evidence less sequence identity. Functional analyses reveal that Sp1 and Sp4 transcription factors are strong activators in mammalian cell lines, whereas the structure and function of Sp3 is more complex. Complicating this further, Sp1 self-interaction and protein-protein interaction between Sp1 family members is well documented. Domain swapping experiments have highlighted important
differences in Sp1 and Sp3 structural elements consistent with the view that interactions with other transcription factors, co-activators and the general transcription machinery may differ between Sp1 family members (117). In the current studies EMSA and functional promoter analysis in endothelial and Drosophila Schneider cells provided clear evidence for the important and complex contributions of Sp1 family members to eNOS promoter activity. PRD I corresponds to a high-affinity Sp1 site [5'-GGGGCGGGGC-3'] located at -104 to -95 (Figure 31). Protein/DNA complexes that formed on an oligonucleotide spanning PRD I (-120/-91) contained Sp1 and multiple variants of Sp3. EMSA and functional studies also suggested the presence of a low-affinity Sp1 site [5'-CCTCCC-3'] at positions -146 to -141 in PRD II. An oligonucleotide spanning this region of PRD II was also recognized by Sp3 variants.

Sp3 is a bifunctional protein that can either activate or repress Sp1-responsive elements in a promoter-dependent context. Stimulation of transcription by Sp3 has been demonstrated for a number of native promoters, sometimes exhibiting synergy with Sp1 (200). This contrasts with other promoters, such as the HIV I LTR, wherein Sp3 acts as an inhibitor of Sp1-mediated activation (199). When Sp1 and Sp3 were expressed concomitantly in cells, Sp3 inhibited Sp1-mediated activation of the HIV-1 LTR (117). This repression was dependent on the DNA binding domain of Sp3. A mutant of Sp3, lacking the COOH-terminal DNA binding domain, did not repress Sp1-dependent transcription suggesting that the inhibitory effect of Sp3 may be a consequence of competition with Sp1 for their common DNA recognition sites. Work from others suggested a unique repressor domain in Sp3 immediately upstream of the DNA-binding domain (200).
Figure 31. Schematic representation of the proximal promoter region of the human eNOS gene.
+1 represents the transcription start site. Narrow arrows indicate the nucleotide position of CpG dinucleotides, while wide arrows indicate Mspl/HpaII sites (5'-CCGG-3') within the proximal promoter region. PRD represents positive regulatory domain.
Newer insight has evolved with the realization that Sp3 mRNA encodes at least three transcription factors, two of which arise from alternate translation initiation sites (160). Studies have revealed three prominent proteins of 115, 80 and 78 kDa that are abundantly expressed in a broad number of mammalian cell types. Each of these variants is recognized by antisera prepared against the Sp3 protein (160). The shorter Sp3 isoforms (78 and 80 kDa) co-migrate as a fast protein/DNA complex and the 115 kDa Sp3 isoform migrates as a slower protein/DNA complex. EMSA results in endothelial cells with PRD I and PRD II probes are consistent with this formulation. Kennet et al (160) concluded that the internally initiated Sp3 isoforms function as potent inhibitors of Sp-mediated transcription, whereas the full-length protein is an activator of transcription. In view of these recent findings the current studies sought to define the relative functional contributions of the varied Sp3 isoforms. We report here that promoter regions of the eNOS gene are cooperatively activated by Sp1 combined with full-length Sp3, both in the presence and absence of Ets-1. Sp3ΔNH2 failed to modulate eNOS promoter activity in Drosophila Schneider cells by itself but potentiated the stimulatory effects of the combined addition of Ets-1, Sp1, and full-length Sp3. This enhancement was observed over a wide range of Sp3ΔNH2 expression. A biphasic effect was seen when Sp3ΔNH2 was co-expressed with Sp1 alone. Curiously, repression was observed in the setting of limiting amounts of the Sp3ΔNH2 mutant. Part of this complexity may be related to the nature of the binding sites in PRD I and II. Others have suggested that multimerization of Sp1 with short and long forms of Sp3 may exert positive or negative effects on transcription depending on the number of Sp1/Sp3 binding sites in the promoter (160). Limiting concentrations of Sp3ΔNH2 protein may inhibit eNOS promoter transactivation by Sp1 through disruption of intermolecular Sp1 interactions involving both PRD I and II. With the addition of increasing amounts of Sp3ΔNH2 protein, this inhibition may be overridden by the formation of transcriptionally active multimeric complexes that involve
interactions between different domains of Sp1 and Sp3ΔNH2. A similar mechanism may be operative when Sp3ΔNH2 is added to threshold amounts of Ets-1, Sp1 and full-length Sp3 proteins. Here, highly coordinated interactions created through protein-protein and protein-DNA interactions creates multimeric nucleoprotein complexes that robustly activate the eNOS promoter in a manner that is not dependent upon a single or more limited repertoire of intermolecular Sp1 interactions.

It is clear that concomitant expression of variants of Sp3 along with Sp1 in endothelial cells adds a level of complexity to understanding the functional role of eNOS PRD I and PRD II. In Drosophila Schneider cells Sp1-mediated stimulation of eNOS transcription is, for the most part, enhanced by Sp3 rather than abrogated. These findings may have relevance to changes in eNOS transcription that attend the varied unique exogenous stimuli to which the vascular endothelium can respond to, especially since the ratio of Sp1 and Sp3 molecules in a cell can vary during differentiation. A representative example is the involvement of Sp3 in Sp1-dependent activation of p21Cαp1 W 1 F1 expression upon keratinocyte differentiation (253). In these cells it is Sp3 that accounts for the induction of expression, not Sp1. Cellular activation mechanisms in vascular endothelium may exert distinct effects on Sp1 and Sp3 structure and function especially since post-translational modification of Sp1 family members is known to exert functional effects on promoter activity (53).

Studies in endothelial and Drosophila Schneider cells indicate a critical role for PRD II in efficient transactivation of the eNOS promoter. Though PRD I is necessary for eNOS activation it is not sufficient, given that mutating 5’- or 3’- regions of PRD II interferes with efficient eNOS transactivation. An interesting architectural feature of PRD II is the presence of many putative
cis-DNA elements (Figure 31). Especially intriguing was an Ets binding site. Ets proteins bind to the invariant core motif [GGAA/T]. The Ets DNA binding domain, which covers approximately 85 amino acids, has no structural homology to other known DNA-binding motifs such as zinc finger, helix-turn-helix or leucine zippers but is sufficient for specific DNA interaction (145). The contribution of Ets family members to gene regulation in vascular endothelium is a newer story. Vascular endothelium is known to constitutively express Ets-1 (110, 336), Ets-2 (336), Erg-1 (110, 162), ERM (110), ER81 (110). Angiogenesis and cytokine-activation represent important changes in endothelial phenotype that are associated with alterations in Ets family member expression and function (142, 336). For example the transcription of Ets-1 and Ets-2 is significantly upregulated in proliferating endothelial cells or after activation by interleukin-1β (162) and TNF-α.

Given EMSA results (Figure 17 and 18) suggesting involvement of Ets family members in nucleoprotein formation upon PRD II we determined whether Ets-1, a major Ets family member in endothelial cells, participated in activation of the eNOS promoter. Results suggested that Ets-1 can cooperate with Sp1 and/or Sp3 in a cooperative fashion in cells known not to constitutively express Ets proteins, namely Drosophila Schneider cells (Figure 20 and 21). This is well illustrated in studies that evaluated interactions between threshold amounts of Ets-1, full-length Sp3 and Sp1. Removing any one of these factors abrogated their activation potential. Mutating any of the activator recognition sites found in PRD I and II also resulted in a marked decrease in functional promoter activity (Figure 21). These results demonstrated the functionally important contribution of the Ets-1 transcription factor in activation of the eNOS promoter. The contribution of Ets proteins in transcriptional regulation often involves participation in protein-protein interactions with other nuclear factors, especially Sp1 family members, so the
interdependence on other transcription factors was not unexpected. For example, the LTR of human HTLV-I contains a Ets-1-responsive element that is dependent on the integrity of an adjacent Sp1 cis-DNA element for synergistic activation of HTLV-I transcription (102). Similarly, the P4 promoter of parovirus minute virus is synergistically transactivated via neighbouring Ets-1 and low-affinity Sp1 sites (96). In this regard, we propose that low- and high-affinity Sp1/Sp3 protein-DNA interactions participate with Ets-1 in activating the proximal eNOS promoter through PRD I and PRD II. This is the most plausible interpretation of the findings reported in the current work. It should be acknowledged that conclusive evidence of Ets-1 binding to PRD II is not provided in the present work given the acknowledged difficulties with Ets family member antisera and cross-competition among Ets binding sites. Moreover, an autoinhibitory domain of Ets-1 can inhibit important protein-protein or protein-DNA interactions in EMSA studies. This has led to the realization that the Ets-1 protein is negatively regulated through conformational changes involving intramolecular interactions (151). Specifically, an inhibitory module allosterically modulates the DNA-binding activity of Ets-1. Activation of Ets-1 requires a conformational change and this autoinhibition of Ets-1 can be relieved by either protein partner(s) or post-translational modifications (151). This concept is relevant to a model discussed below.

With the exception of GABPalpha, Ets family members bind the core motif as a monomer. Approximately 10 bp of DNA sequence containing the Ets core motif determines which Ets family member will bind and hence specificity. Sequence comparison of PRD II of the eNOS gene at -132/-122 [5'-A C A G G A A C A-3'] with previously identified Ets binding sites revealed near identity with the HIV-2 LTR enhancer [5'-A C A G G A A C A G-3'] which is known to bind Elf-1 (179) (Table IV). Elf-1 is a transcription factor previously implicated in the
Table IV. Comparison of known Elf-1 binding sites

<table>
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<tr>
<th>eNOS (PRD II)</th>
<th>A</th>
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<th>A</th>
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<td>A</td>
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<td>HTLV-I LTR (PuB1)</td>
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<td>HTLV-I LTR (PuB2)</td>
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<td>consensus derived from binding site selection</td>
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inducible activation of genes in mature T-cells. For example, Elf-1 participates in the inducible regulation of CD4, granulocyte-macrophage colony-stimulating factor and IL-2 receptor α chain (IL-2Rα) following T-cell activation (306, 345). Elf-1 plays a role in the developmental regulation of the terminal transferase gene in early lymphocyte development (83). Elf-1 is also required for inducible T-cell trophic viruses including HIV-2 and HTLV-1 (55, 179). Elf-1 is highly expressed in B cells where it participates in the regulation of a variety of genes, including IgH, lyn and lck (232). As a rule, the Ets family of transcription factors is widely distributed in varied tissues and cell types. Therefore, the cell restricted expression of Elf-1 contrasts with the expression of most Ets family members. Only a few Ets family members demonstrate a cell type-specific expression pattern (145). For example, PU.1 is B cell and macrophage-restricted and ESE-1 is primarily found in epithelial cells (233). Elf-1 protein expression was thought to be relatively restricted to lymphoid and myeloid cells (306). The present work demonstrates that Elf-1 is constitutively expressed in vascular endothelium and can bind to PRD II and consensus HIV-2 LTR sequences. Elf-1 was not previously appreciated as playing a role in the control of gene expression in vascular endothelium. It is of interest that novel Elf-1-like proteins continue to be cloned and characterized. Recently NERF (232), Elf-2 (339), Elf-3 (233, 234, 313), Elf-5 (360) and MEF (215) were identified. These proteins contain domains that are structurally similar to the Ets binding domain of Elf-1. Therefore, we cannot exclude the possibility that a protein antigenically-related to Elf-1 exists in endothelial cells.

An intriguing facet of Elf-1 biology is that the consensus motif for optimal Elf-1 binding [5'-A A/t C/a C C G G A A G T a/g/c-3'] determined by a binding site selection method does not conform to known functional Elf-1 sites (Table 4) (147). Conversion of a naturally occurring, low-affinity Elf-1 site in the IL-2Rα promoter to an optimal site resulted in a decrease in the
ability of Elf-1 to induce transcription (147). Therefore, high affinity Elf-1 sites may lack sufficient biological specificity even though Elf-1 is more discriminatory than Ets-1 in binding site selection (32). The absence of naturally occurring high affinity Elf-1 sites in Elf-1 regulated genes may represent a mechanism to achieve greater inducibility and may emphasize the importance of accessory proteins in modulating Elf-1 binding and action. One exception to this generalization has been recently provided. Elf-1 plays an essential role in the trans-activation of the TCR-zeta subunit, a constitutively expressed T-cell-specific gene, through a site that is the best match with the optimal Elf-1 consensus of any known mammalian Elf-1 binding site (264). This suggests that the requirement for post-translational modulation of Elf-1 or other inducible trans-factors in Elf-1-mediated trans-activation may decrease as the affinity of the Elf-1 site increases. In the current work we report that (i) the Elf-1 binding site in PRD II does not conform to the optimal Elf-1 consensus sequence. (ii) Elf-1 protein minimally enhanced eNOS promoter activity in Drosophila Schneider cells by itself. (iii) Elf-1 was a potent inhibitor of Sp1-mediated transactivation in Drosophila Schneider cells. (iv) Elf-1 did not repress eNOS promoter activity in the presence of Ets-1 in Drosophila Schneider cells. (v) PRD II nucleoprotein complexes in BAEC contain Elf-1, and (vi) augmented Elf-1 expression in BAEC enhanced eNOS promoter activity and this increase was dependent upon the Ets binding site in PRD II.

In general, Elf-1 serves as a transcriptional activator for many inducible T-cell genes. These promoters often contain adjacent or overlapping binding sites for the Elf-1 and NF-κB/NFAT families of transcription factors. For example, Elf-1 participates in the inducible expression of the HIV-2 LTR via Ets binding sites and a neighbouring NF-κB/NFAT binding site (179). Elf-1 functions cooperatively with the NF-κB family of transcription factors to activate transcription
of the HIV-2 LTR during T-cell activation. Both proteins are necessary for this activation. Overexpression of Elf-1 alone fails to activate the HIV-2 LTR. Also, a dominant-negative mutant of NF-κB p50 that binds DNA but fails to interact with Ets proteins inhibits the synergistic activation of the HIV-2 enhancer by NF-κB (p50 + p65) and Ets family members (17). As a further example, cell-type specific expression of the IL-2Rα promoter in T-cells also involves, in part, synergistic interactions between Elf-1, HMG-I(Y) and NF-κB family proteins (148). The current studies demonstrated that Elf-1 exerts a repressive effect on Sp1-mediated promoter activation in Drosophila Schneider cells but enhanced activity in endothelial cells. Further studies will be necessary to understand why the contribution of Elf-1 to transcriptional regulation of the eNOS gene differs between Drosophila Schneider cells and endothelial cells. Clearly some members of the Ets family of transcription factors exhibit strong transcriptional repressor activity, such as ERF (285). It may be possible that Elf-1 requires a co-activator in order to demonstrate activation potential. Also, activation of MAPK signal transduction pathways results in changes in the activity of many ETS-domain transcription factors (92). Therefore, a further possibility may be a requirement for post-translational modification of Elf-1 for it to function as an activator, much like BOB kinase activity is required for BOB co-activator to functionally activate Oct1 or Oct2 in B and T-cells (112). It is plausible that such a pathway(s) may not be functional in Drosophila and Elf-1 protein may be in a conformation that sterically inhibits its capabilities of functioning as an activator. Finally, it is known that Elf-1 forms complexes in T-cells with the underphosphorylated form of the retinoblastoma protein (Rb) both in vitro and in vivo. Overexpression of unphosphorylated Rb inhibits Elf-1-dependent transcriptional activation in T-cells (325). After T-cell activation, phosphorylation of Rb leads to the release of transcriptionally competent Elf-1 (325). This coordinated regulation of Elf-1 may be deficient in Drosophila Schneider cells.
EMSA analyses with PRD II oligonucleotides demonstrated binding of recombinant YY1 and the existence of YY1 in PRD II nucleoprotein complexes (Figure 17D), likely at position -121 to -117 (Figure 31). YY1 is a ubiquitously expressed 65 - 68 kDa GLI-Kruppel-related protein that contains four C2H2-type zinc fingers at the COOH-terminus (224) and is a multifunctional transcriptional regulator. Depending on promoter and cellular context, it can activate or repress transcription. YY1-binding proteins so far identified include Sp1 (284), the oncoprotein c-myc, cyclophilin A, FK506-binding protein, p300. ATF/CREB and the mammalian homologue of RPD3 (349). Given this complexity, future studies will be needed that address the functional contributions of YY1 to eNOS promoter structure and function in episomal and chromatin-based assays. Our current working model is that YY1 will activate transcription given that its consensus binding site is an activator site located in 3'-regions of PRD II. It is of interest that YY1 has been shown to physically interact with Sp1 (284) and tightly clustered YY1 and Ets binding sites have been functionally characterized in other genes, such as the cytochrome c oxidase subunit VIIc (NRF-2/NERF-2) or human P19 parvovirus (GABPre) promoter (280, 318). In the case of the eNOS promoter, the YY1 and Ets binding sites are separated by 4 nt in PRD II. It is presumed that the four zinc fingers of YY1 interact with 12 nt of the bottom strand, with the core motif positioned at the center (280). This may indicate that the binding of one factor would sterically hinder the binding of the other. One mechanism of YY1 repression involves preventing the binding of activator proteins via overlapping binding sites. For example, overlapping binding sites have been observed for YY1 and NF-κB in the serum amyloid A1 gene promoter. YY1 can also enhance the binding of an activator to an adjacent binding site by inducing DNA binding thereby facilitating the interaction of an activator with the basal transcriptional machinery (164). Perhaps in the absence of YY1, Elf-1 is unable to interact with the basal transcriptional
machinery and thus requires the presence of YY1 to facilitate this interaction. It is of interest that the eNOS gene does not evidence a canonical TATAA element (254) given that YY1 has also been implicated in the formation of preinitiation transcription complexes independent of TATA-binding protein (317).

The current work demonstrated that MAZ (myc-associated zinc finger protein) participates in protein-DNA and protein-protein interactions in PRD II regulatory regions of the eNOS promoter. In Drosophila Schneider cells MAZ exhibits a negative effect on eNOS promoter activity and this repressor activity is especially dependent on the Ets site in PRD II. MAZ has received increasing attention for its protean roles in gene regulation: transcription initiation, interference and termination (33). MAZ is especially important in TATA-less promoters (243). Particularly well-studied examples of MAZ involvement in transcriptional initiation include c-myc, the adenovirus major late promoter, the serotonin 1a receptor and CD4 (33, 80, 243). An important facet of MAZ function is the participation of partner proteins. MAZ polypeptide contains multiple functional domains in addition to the 6 structurally important zinc-fingers (His₂Cys₂) (33). Functional interactions between MAZ and Spl occur in a number of genes, including c-myc (74, 243). In the case of CD4, enhancer activity during development is critically dependent upon MAZ and an Ets consensus site that binds Elf-1 (80, 345). The functional contributions of MAZ are complex considering that not only is MAZ necessary for efficient initiation and transcriptional elongation of c-myc P2 promoter transcripts, through the ME1al site in the P2 promoter, but MAZ and ME1al-like binding sites are also involved in the transcriptional pausing/attenuation of the c-myc gene, and also the human complement C2 gene (33). In certain respects the human eNOS promoter evidences sequence similarity with the human c-myc promoter: tandem CT elements in the P1 promoter and the single functional CT
(ME1a1) element of the P2 promoter are reminiscent of the numerous CT elements in the eNOS promoter. Though multiple potential MAZ-binding CT elements were identified in the eNOS promoter at -191, -146, -99, -75, -62, and -47, the evidence in the current work highlighted that the repressive effect of MAZ on eNOS promoter activity in Drosophila Schneider cells is likely mediated via PRD II. The complexity that has emerged from studies assessing the contributions of MAZ to regulation of c-myc expression suggests that further nuances of MAZ and eNOS expression will evolve, perhaps also involving both regulation of transcriptional initiation and pausing.

In summary, these studies demonstrate an unexpected complexity in the regulation of eNOS gene expression. We posit that Sp1, variants of Sp3, Ets-1 and Elf-1 are important components of the eNOS promoter structure, which are responsible for the activation of eNOS transcription in endothelial cells (Figure 31). Cooperativity between these trans-acting factors is likely to require multiple protein-protein and protein-DNA interactions (79, 305). Distinct domains of transcription factors are responsible for protein-protein and protein-DNA interactions. For example, the Ets family is characterized by the conserved DNA-binding ETS domain (157, 230). The ETS domain is necessary and sufficient for these proteins to bind specifically to DNA in vitro (103, 145, 185). Recently, nuclear magnetic resonance structural analyses have determined that the ETS domain displays a winged helix-turn-helix motif (77, 78). Domain swapping experiments involving fusion of deletion mutants of various Ets factors to heterologous DNA-binding domains have been performed to map the activation domains of Ets proteins. For example, three transactivation domains (RI, RII and RIII) have been identified in the amino-terminal portion of Ets-1 (277). The transactivation domains of PU.1 have also been mapped and consist of a Gln-rich region spanning amino acids 74-93 and an acidic region spanning amino
acids 1-165 (90). Similarly, the transactivation domain of Elf-1 has been mapped to the N-terminal 86 amino acids (36). Certain domains of the Ets protein are also responsible for protein-protein interactions. For example, the pointed domain, also referred to as the helix-turn-helix region, participates in protein-protein interactions (295). The pointed domain, a highly conserved 80 amino acid region, occurs in many Ets proteins, including Ets-1, Ets-2 and Elf-1 (295). The pointed domain was initially proposed to mediate protein-protein interactions between members of the Ets family of transcription factors (295). For example, the TEL pointed domain causes self-association (174), however the pointed domains of Ets-1, Erg and GABPα do not cause self-association (152)(173).

Thus Sp1. variants of Sp3, Ets-1 and Elf-1 functionally cooperate to present to the basal transcriptional machinery a biochemical interface that is highly efficient in transcription initiation. In the current work, coexpression of Sp1, Sp3 and Ets-1 in Drosophila Schneider cells enhanced transcription of eNOS promoter/reporter constructs compared to each factor alone. Mutating activator recognition sites for these factors, or removal of any of these factors, abolishes this cooperativity. This suggests that activation of the eNOS promoter is dependent upon protein-protein interactions between these factors as well as interactions between the trans-factors and their corresponding cis-elements in PRD I and PRD II. Sp1 is known to recruit and physically interact with itself and varied trans-acting factors: Sp3, AP-1, GATA members, NF-kB, Ets-1, among others. Based upon this background a model can be proposed. Following an initial binding of Sp1 to a high-affinity element in PRD I, other Sp1 molecules and variant Sp3 proteins are recruited through binding or tethering and interact with low-affinity elements in PRD II. DNA binding domains of Sp1 family members have been reported to unwind DNA as well as bend DNA (293) upon binding. These DNA deformations may be important in determining
overall binding affinities as well as influencing binding site preferences for neighbouring sites, but are not by themselves sufficient for transactivation (293). These changes may also enhance the transactivation potential of Sp1 family members. Ets factors also interact with other proteins to form either multi-subunit complexes or ternary complexes that are stable only in the presence of DNA. Therefore, an initial recruitment of Sp1 may also facilitate the binding of Ets-1. Domain interactions between Sp1 and Elf-1 proteins has not yet been described, nor has an interaction between Elf-1 and Ets-1 proteins. We therefore suspect that post-translational modifications of Elf-1 figure prominently in determining the functional contributions of Elf-1 to eNOS promoter function in endothelial cells, likely by modulating binding site affinity. The prior findings that MAZ is capable of inducing a 72° bend in the DNA helix and that YY1 has also been shown to induce bending of DNA (224) indicate that protein-DNA and protein-protein interactions on the eNOS promoter may be modulated by complex architectural features. In this regard, future detailed biochemical analysis of protein-DNA and protein-protein interactions on the eNOS promoter will be needed to substantiate this model further. For example, DNase I footprinting studies and gel shifts on natural promoters will need to be performed to confirm the cooperative interaction of the trans-factors identified. As well, point mutations and methylation interference studies would also support these findings. It will also be necessary to determine what alterations occur in the complex eNOS promoter structure and function in conditions known to be associated with biologically important alterations in eNOS mRNA expression. Especially relevant to health and disease are alterations in fluid shear stress and atherosclerosis.
CHAPTER IV

THE ROLE OF DNA METHYLATION IN THE CELL-SPECIFIC GENE EXPRESSION OF HUMAN eNOS

Portions of this chapter have been submitted as:
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Gnanapandithen, K., Zahariadis, G. and Marsden, P.A.
CHAPTER IV. THE ROLE OF DNA METHYLATION IN THE CELL-SPECIFIC GENE EXPRESSION OF HUMAN eNOS

IV.1 OBJECTIVE AND AIMS

An intriguing aspect of eNOS gene expression is its relative restriction to the vascular endothelium. Possible transcriptional regulatory mechanisms involved were examined through a series of non-chromatin-based transient transfection assays with eNOS promoter/reporter constructs in a variety of endothelial and non-endothelial cells. Interestingly, robust eNOS promoter activity was demonstrated in a number of non-endothelial cell types in which no appreciable eNOS mRNA transcripts were detected by RNase protection and Northern blot analysis (Chapter II. Figure 14A and data not shown). As shown in the next chapter, there exists a high degree of sequence conservation with respect to trans-factor binding sites in the eNOS promoter across species. In vivo eNOS studies using a murine model supports this model (304). A promoter/reporter insertional transgene containing the native murine eNOS promoter (-5200/+28) directed transcription of nuclear-localized β-galactosidase in an endothelial cell-specific fashion. This in vivo expression profile was uniform across multiple founders and not dependent upon the site of integration into mouse genomic DNA. The findings of this work demonstrated that murine eNOS genomic regions spanning -5200/+28, relative to the transcription start site, directed expression of a reporter construct in a fashion that recapitulated the known expression profile of eNOS mRNA and protein. These results suggest that chromatin-based mechanisms actively repress native eNOS gene expression. Therefore, our work has identified a discrepancy between the expression patterns of episomal versus chromatin-based eNOS promoter-reporter
constructs in non-endothelial cell types. We posit that epigenetic contributions play a role in the cell-specific expression of eNOS. Specifically, we posit that demethylation of the eNOS 5' flanking regions occurs in an endothelial cell-specific fashion.

IV.1.1 OBJECTIVE

The objective is to determine whether DNA methylation plays a role in the transcriptional regulation and endothelial cell-specific expression of human eNOS.

IV.1.2 SPECIFIC AIMS

1. To examine the methylation pattern of the eNOS gene in endothelial cells versus non-endothelial cells using the sodium bisulfite genomic sequencing method.

2. To evaluate the functional consequences of CpG methylation of the human eNOS 5' flanking region, using transient transfection analysis of promoter/reporter constructs in BAEC and Drosophila Schneider SL2 cells.

3. To study the effects of 5-azacytidine induced hypomethylation on eNOS mRNA expression in endothelial and non-endothelial cells.

IV.2 HYPOTHESIS

Endothelial cell-specific expression of eNOS is regulated, in part, through epigenetic contributions.
**IV.3 MATERIALS AND METHODS**

*Genomic DNA Isolation.* Genomic DNA was isolated from various human cell types including HUVEC, human microvascular endothelial cells (HuMVEC), human vascular smooth muscle cells (HuVSMC), HeLa, HepG2, JEG-3, TGW, and human peripheral blood leukocytes as described previously (12).

*Sodium Bisulfite Genomic Sequencing.* Genomic DNA isolated from HUVEC and non-endothelial cells (5 μg) was digested with *Bam*H1 and then subjected to the sodium bisulfite treatment as previously described (56). Sodium bisulfite converts cytosine to uracil, while leaving 5-methyl-cytosine unchanged. The uracil residues are then PCR-amplified to thymines. PCR primers for amplicon in the regions of interest were specifically designed to the sodium bisulfite-modified template. The bisulfite-treated DNA (150 ng) was subjected to 35 cycles of PCR amplification, followed by another 35 cycles of nested PCR amplification. The final products were either directly sequenced by the dye terminator fluorescence sequencing method (ABI Prism 377) or subcloned using the TA cloning kit (Invitrogen) to yield individual strands, followed by sequencing. Over 300 subclones were screened for each cell type and ten positives were randomly sequenced to yield the final % methylation results. Briefly, the sodium bisulfite modification is as follows. The *Bam*H1-digested DNA was first denatured with 0.3M NaOH for 15 minutes at 37°C since sodium bisulfite works on single stranded DNA. The denatured DNA was then treated with 3.1 M sodium bisulfite and 0.5 mM hydroquinone (Sigma Chemical), overlaid with mineral oil, and incubated at 55°C for 16 h in the dark. The DNA was recovered by snap freezing and free bisulfite was removed using the Promega Wizard DNA clean-up desalting column (Promega) followed by an incubation in 0.3 M NaOH for 15 minutes at 37°C to denature
and to remove the -SO₃ adduct from the uracil bases prior to the PCR reaction. Finally, the DNA was neutralized by NH₄OAc, precipitated and stored at -20°C.

**Primers and Oligonucleotides.** All oligonucleotides were synthesized using the Beckman Oligo 1000 DNA synthesizer (Beckman Instruments, Fullerton, CA). The primer sets used in sodium bisulfite genomic sequencing were designed to the 5'-flanking region, exon 1, and the 3'-GC rich region of the eNOS gene according to the criteria previously described (56), using the Oligo 4.0 software (Table V). The primer sets are denoted Sodium Bisulfite Polymerase Chain Reaction (SBPCR) followed by the 5' position of the PCR amplicon relative to the eNOS transcription start site (+1). Primer set SBPCR-249 spans a 476 bp region of the eNOS core promoter from -249 to +227. SBPCR-132 spans a 382 bp region from -132 to +234. These primers were designed to span a 446 bp region in exon 24 of eNOS from +19767 to +20212. SBPCR-183 was a primer internal to SBPCR-249 to increase the sensitivity of the PCR reaction. Primer set SBPCR-183 produces an amplicon of 381 bp spanning a region from -183 to +208. A set of nested PCR primers was specifically designed to probe the methylation status of the functionally important regions in the eNOS promoter, namely positive regulatory domains I and II (PRD I, II) (Figure 32). SBPCR-340 is a set of primers that spans a genomic region of 609 bp from -340 to +269. SBPCR-302 is the internally nested primer set to SBPCR-340. SBPCR-302 primers span a 358 bp region from -302 to +56. SBPCR+19767 primer set spans a 446 bp region of exon 24 and was designed to probe the methylation status of the 3' GC-rich region of the eNOS gene. SBPCR+19642 (868 bp amplicon) is a set of primers internally nested to SBPCR+19441 (567 bp amplicon) and they also were designed to amplify the GC-rich region in exon 24.
<table>
<thead>
<tr>
<th>PRIMER</th>
<th>SEQUENCE</th>
<th>STRAND</th>
<th>LENGTH (nt)</th>
<th>LOCATION</th>
<th>T&lt;sub&gt;s&lt;/sub&gt; (°C)</th>
<th>AMPLICON SIZE (bp)</th>
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<td>5'- TTG TAG GGA AAG GTA TAT AGG GGT GA -3'</td>
<td>sense</td>
<td>26</td>
<td>core promoter</td>
<td>52.3</td>
<td>609</td>
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<td></td>
<td>5'- ACC CTT ATT ACC CAC CTA CTC CTA ACT -3'</td>
<td>antisense</td>
<td>27</td>
<td>-340 to +269</td>
<td></td>
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<tr>
<td>SBPCR-302</td>
<td>5'- GTG TGG TGT TAT ATT ATA GAA GGA TT -3'</td>
<td>sense</td>
<td>26</td>
<td>core promoter</td>
<td>48.3</td>
<td>358</td>
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<tr>
<td></td>
<td>5'- CTC TAC TAC CTA CTC CAA CAA AAC CC -3'</td>
<td>antisense</td>
<td>26</td>
<td>-302 to +56</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SBPCR-249</td>
<td>5'- GGA TAT TTG GGT TTT TAT TTA TTA GTT TTA -3'</td>
<td>sense</td>
<td>30</td>
<td>core promoter</td>
<td>52.1</td>
<td>476</td>
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<tr>
<td></td>
<td>5'- TAA CAC CAC CCT TAT TAC CCA CCT ACT -3'</td>
<td>antisense</td>
<td>27</td>
<td>-249 to +227</td>
<td></td>
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<tr>
<td>SBPCR-173</td>
<td>5'- AGT GGG CGT GGA GTT GAG GTT TTA GA -3'</td>
<td>sense</td>
<td>26</td>
<td>core promoter</td>
<td>52.5</td>
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<td>27</td>
<td>-173 to +208</td>
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<td>SBPCR-132</td>
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<td>antisense</td>
<td>27</td>
<td>-132 to +234</td>
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Figure 32. Mapping of CpG dinucleotides in the proximal promoter region of the human eNOS gene. +1 represents the transcription start site. Narrow arrows indicate the nucleotide position of CpG dinucleotides, while wide arrows indicate MspI/HpaI sites (5′-CCGG-3′) within the proximal promoter region. PRD represents positive regulatory domain.
**Expression Constructs.** The MeCP-2 cDNA was kindly provided by A. Bird, Institute of Cell and Molecular Biology, University of Edinburgh, United Kingdom and was previously described (182). For construction of pPacUMeCP-2 expression construct, rat MeCP-2 was partially digested with SacI. The linearized plasmid was isolated by preparative gel electrophoresis and further subjected to EcoRI complete digestion to yield a 1.8 kb fragment. This fragment was also isolated by gel electrophoresis, blunt-ended with Klenow. The resulting MeCP-2 ORF was cloned into the BamHI sites of pPacUO. All other constructs are described in Chapters II and III.

**Methylation of Promoter/Reporter Constructs.** The eNOS promoter/reporter construct (pGL2-1193/+109 eNOS-luciferase reporter) was in vitro methylated by various methylases including HpaII (CmCGG), MspI (mCCGG), and SssI (mCG) as previously described (266). The construct was also mock-methylated where the reaction did not contain any methylase. High and low-density methylation constructs were prepared. For preparation of the SssI low-density methylation construct, only 1/5 of the SssI methylase used to methylate the SssI high-density methylation construct was used in reaction. The methylation status of the constructs was verified using methylation-sensitive isoschizomers HpaII and MspI. The methylated and mock-methylated constructs were phenol/chloroform purified and subsequently precipitated before transfections.

**5-azacytidine Treatment.** HUVEC, HeLa, HepG2, JEG-3, and TGW were seeded at a density of routine densities (3-4 x 10^5 cells/ml) and treated with 5 μM 5-azacytidine for 7 days (193). Cells were treated and fed every 48 hrs subsequent to seeding. The similar number of untreated plates was cultured in the same fashion concurrently as controls.
**Total RNA Extraction.** Total RNA was extracted from various human cell types including HUVEC, HeLa, HepG2, JEG-3, TGW, and human peripheral blood leukocytes as described previously (203).

**RNase Protection Assays.** 50 μCi [α-32P]CTP (800 Ci/mmol) were used to generate an internally labeled antisense riboprobe in an *in vitro* transcription reaction using T7 RNA polymerase (Promega) and 1 μg of EcoRI linearized template DNA spanning the 292 terminal nucleotides of the human eNOS cDNA. Specific activity of the probe is 1.5 x 10⁹ cpm/μg. A labeled RNA ladder was synthesized similarly using the Century Marker DNA template (Ambion Inc). DNA templates were removed by DNase digestion followed by gel purification on a 6% acrylamide / 8 M urea gel. Eluted probe was hybridized in solution with 10 μg of total cellular RNA from HUVEC, HeLa, HepG2, JEG-3, and TGW overnight at 45°C and subjected to RNase A and T1 digestion at 25°C for 30 minutes. The RNase digestion was inactivated according to RPA II kit (Ambion Inc. Austin, Texas) instructions and the RNA was precipitated. The resulting samples were electrophoresed through a denaturing 6% acrylamide / 8 M urea gel, followed by autoradiography and phosphorimaging.
IV.4 RESULTS

IV.4.1 SODIUM BISULFITE GENOMIC SEQUENCING RESULTS (46).

The determination of the methylation status of the eNOS gene constitutes the first step in deciphering the possible relation between the cell-specific expression of eNOS and DNA methylation. To date, the most robust assay for studying the methylation pattern of DNA is the sodium bisulfite genomic sequencing method (56, 57, 95, 123, 274). It allows the positive identification of 5-meC in the DNA sequence rather than a negative or lack of result identification. Using single-stranded DNA as substrates, sodium bisulfite deaminates cytosines to uracils (56). This reaction is highly specific for single stranded DNA templates and cytosines. Although 5-meC also reacts with the bisulfite, the reaction is extremely slow and the equilibrium favors 5-meC (56) (Figure 33). Therefore, cytosine and 5-methylcytosine can be distinguished.

First, sodium bisulfite is added to the carbon 5-6 double bond of cytosine, forming sulfonated cytosine. The extent of adduct formation is controlled by pH, bisulfite concentration, and temperature (57). The second step involves the hydrolytic deamination of cytosine sulfonate to give a uracil-bisulfite derivative, uracil sulfonate. Finally, the removal of the sulfonate group is carried out by an alkali treatment, giving uracil as the end product.

The overall method involves the denaturation of genomic DNA to yield single stranded templates (Figure 34). After denaturation, the genomic DNA is subjected to the bisulfite treatment. Opposite strands of the treated DNA are no longer complementary, and can therefore be amplified by PCR in two separate reactions, using primers specific to each of the modified strands (C to U) (56). In each PCR product, all the U's (converted C's) will be amplified as T's,
Figure 33. Schematic representation of the chemical deamination reaction of cytosine by sodium bisulfite. The three steps include: 1. sulfonation, 2. hydrolytic deamination, and 3. alkali desulfonation.
Figure 34. Overview of the sodium bisulfite genomic sequencing method. Bisulfite sequencing of a schematic DNA. (a) and (b) will identify the two original strands of the target DNA, for they will no longer be complementary after the bisulfite treatment. The major steps in the bisulfite sequencing protocol include denaturation of the DNA strands, bisulfite modification, PCR amplification, and sequencing.
and the only remaining C's will represent a 5-meC in the original DNA molecule. Following the initial PCR amplification, we found that a nested PCR reaction can be performed to increase the specificity and product yield. Finally, sequencing the PCR product will identify all the methylated C's in the original DNA sequence. Two approaches were used for sequencing: (1) direct sequencing of the PCR product, using fluorescence-based methodologies (ABI Prism 377 sequencer), to give an average sequence and methylation pattern of each strand of DNA, and (2) subcloning (TA cloning) and sequencing for the status of individual DNA strands (95).

The most critical step in the bisulfite genomic sequencing protocol is the design of primers to the deaminated DNA. The primers are designed to favor the amplification of fully bisulfite-modified DNA from a mixture, which may contain some partially deaminated strands. The following criteria are important for the success of the final amplification step (56). The primers should be 25 to 30 bp in length. They should be designed to an evenly C-rich DNA region. This region of the DNA should contain a minimum number of CpG dinucleotides since the methylation status of the C's is unknown. It is difficult to predict whether the C should remain a C or whether it should be assumed to have been changed to a T. Though newer methods selectively exploit differential PCR amplification based upon CG- or TG-containing PCR primers (123), it is best to avoid such a scenario. In addition, the primer pair should show limited internal complementarity and limited complementary sequences between the pair.
IV.4.1.1 Analysis of CpG sites in the Proximal Promoter Region of Human eNOS in Endothelial vs. Non-Endothelial Cell Types (46).

To determine a possible role for DNA methylation in the cell-specific expression of eNOS, the methylation status of cytosine residues in CpG dinucleotides within the proximal promoter region (see Figure 32) of eNOS were examined in endothelial and non-endothelial cell types (Figure 35). The methylation pattern of nine CpG doublets within the proximal promoter between nucleotide position -209 and -51, relative to the transcription start site, was determined by the sodium bisulfite genomic sequencing method (Figure 36 and 37). Since this method converts cytosines in each strand of the DNA, rendering the two strands no longer complementary after treatment, it is possible to design PCR primers to either of the modified strands in order to examine the methylation status of this genomic region. Primers were specifically designed to the top converted strand (Table V). Primer set SBPCR-173 produces an amplicon of 381 bp spanning a region from -173 to +208. Sequencing of a subcloned PCR product revealed that the bisulfite modification had gone to completion. In total, more than 80 subcloned PCR products (at least 10 for each cell type), each containing approximately 80 to 100 C’s, were sequenced. Of the 80,000 C’s that were examined, less than 50 C’s, representing merely 0.06% of the whole cytosine pool, were not found to be modified. Analysis of the converted bottom strand was also performed, confirming these results (data not shown).

This method allowed the detection of differential methylation between endothelial and non-endothelial cells (Figure 35) as shown by the conversion of all the C’s to T’s in the core promoter region -199 to -156 in the endothelial cell type while all the C’s in the non-endothelial cell type were converted except for the four CpG doublets in the region. Sequencing the PCR product
Figure 35  The sodium bisulfite sequencing method was used to examine the methylation patterns of the eNOS gene in endothelial and non-endothelial cell types. This is a representative sodium bisulfite genomic sequencing results of the human eNOS proximal core promoter of endothelial and non-endothelial cell types. All the cytosine residues (C) in the endothelial genomic DNA have been converted to thymine residues (T). All the C’s in the non-endothelial genomic DNA also converted to T’s, except for four in the CpG doublets at positions -197, -194, -171, and -167 relative to the transcription start site.
Figure 36. Summary of sodium bisulfite genomic sequencing results of subcloned PCR products in endothelial vs. non-endothelial cells. CpG dinucleotide position in the eNOS promoter are indicated on the X-axis.
Figure 37. Summary of sodium bisulfite genomic sequencing results of subcloned PCR products in endothelial vs. non-endothelial cells (cont'd).
(primer set SBPCR-302) directly initially yielded an average methylation status of six CpG doublets in the core promoter region -171 to -51 in the various cell types. The results revealed that none of the cytosines in the six CpG doublets were methylated in HUVEC and in HuMVEC; however, all six were found to be methylated in HuVSMC, TGW and PBL (data not shown). In addition, there was a varying degree of methylation across the six doublets in HeLa, HepG2, and JEG-3. It is apparent that the methylation status of eNOS is drastically different between HUVEC and the non-endothelial cell types.

Subcloning the SBPCR-302 PCR product and sequencing of each subclone yielded the methylation status of the nine doublets from the -209 to -51 region in an individual original DNA strand. Ten subclones of each cell type were analyzed. Each graph summarizes the percentage of subclones (out of a total of ten) that were methylated at each of the given position of CpG doublet within the -209 to -51 region in the eNOS proximal promoter (Figure 36 and 37). The average of these ten individual subclones mirror average methylation status determined by the direct sequencing approach. The nine CpG doublets were all unmethylated in HUVEC and HuMVEC while they were almost completely methylated in TGW, peripheral blood leukocytes and HuVSMC (Figure 36 and 37). Moreover, in HeLa and HepG2, there seems to be a region of especially low methylation density in the -100 region surrounded by upstream adjacent sites that were methylated to a much higher extent. This has been termed a hypomethylation footprint. Specifically, in HepG2, the -95 and -100 sites were only 10% methylated; however, merely 40 bp downstream is the -51 site which was found to be 60% methylated while the -167 site was found to be 90% methylated. Also, the -95 and -100 sites in HeLa were 20 and 10% methylated, respectively, but the -137 site, which is only some 40 bp away, was found to be 80% methylated and the downstream -51 site was found to be 30% methylated. The nine doublets in JEG-3 were almost fully methylated; however, the CpG at -209 displayed an unusually low methylation.
density. Furthermore, analyses of the methylation status of the single original strands from the non-endothelial cell types revealed that none of the individual strands was completely hypomethylated, indicating that the hypomethylation at certain sites is not a mere reflection of the hemi-methylated status of newly replicated DNA. These data confirmed that there is a significant difference between the methylation status of the genomic DNA representing the eNOS proximal promoter in endothelial cells (HUVEC and HuMVEC) compared to genomic DNA isolated from the non-endothelial cell types examined.

IV.4.2 TRANSIENT TRANSFECTION FUNCTIONAL STUDIES IN BAEC AND DROSOPHILA SCHNEIDER SL2 CELLS

IV.4.2.1 DNA methylation inhibits eNOS promoter activity in bovine aortic endothelial cells (46).

To evaluate the functional consequences of CpG methylation in the eNOS promoter sequence, promoter/reporter constructs containing the 5'-flanking region of the human eNOS gene were in vitro methylated using various methylases and were subsequently transiently transfected into bovine aortic endothelial cells (BAEC). The maximally active promoter/reporter construct pGL2-1193/+109 was methylated using the SssI CpG methylase, which specifically methylates C's of CpG dinucleotides (27, 171) for transfection studies in BAEC. A high dose (SssI high) and a low dose (SssI low: 1/5 of the concentration of SssI high) of the SssI methylase as well as methylation by the HpaII (methylates 5'-C\textsuperscript{m}CGG-3') and MspI methylases (methylates 5'-'\textsuperscript{m}CCGG-3') were used to generate constructs with differing methylation density. The extent of methylation was verified by comparing the patterns MspI and HpaII restriction digests. SssI
high-density methylation had no effect on restriction endonuclease MspI as expected, but it completely inhibited HpaII digestion. SssI low methylation density also had no effect on MspI digestion; however, it allowed only partial digestion by HpaII. Moreover, HpaII methylation also mimics the effects of low-density methylation and it completely abolishes HpaII cutting. The difference in HpaII digestion between SssI low and HpaII methylated constructs indicate that although the methylation density may be similar between the two constructs, the specific sites of methylation differed, as only 29 of the 310 CpG dinucleotides are 5'-CCGG-3' sites. A mock-methylated construct (where no SssI methylase was added) was transfected as a control.

Results demonstrated that methylation of the pGL2-1193/+109 promoter/reporter construct had a drastic effect on eNOS promoter activity in BAEC (n=3, triplicate determinations). eNOS promoter activity was completely abolished by high-density methylation (SssI high) compared to the mock-methylated construct (Figure 38). Moreover, the two constructs with low-density methylation (SssI low and HpaII) yielded different results. The eNOS promoter activity of the HpaII construct is merely 1% that of the mock-methylated construct while the activity of the SssI low construct is almost 20%. The difference in the promoter activity between the two low-density methylation constructs suggests that specific sites rather than mere density of methylation may be critical. Lastly, MspI methylation did not have any significant effect on eNOS promoter activity in BAEC, demonstrating the exquisite nature of site specific methylation patterns in the control of gene expression. Taken together, these results imply that DNA methylation may play an important role in the transcriptional repression of the human eNOS gene.
Figure 38. The effects of DNA methylation and methylation density on eNOS promoter activity in bovine aortic endothelial cells (BAEC). SssI high-density methylation indicates that the promoter/reporter construct was methylated with SssI methylase at a high density. SssI low-density methylation indicates that 30% of the CpG sites were methylated. HpaII-methylation represents the construct that was methylated with the HpaII methylase. MspI-methylation represents the construct that was methylated with the MspI methylase. Promoter activity as assessed by luciferase readout was normalized for β-galactosidase activity as well as protein concentration. The data represent the mean ± S.E.M. Shown is a representative experiment (triplicate determinations), n=3. * indicates that the promoter activity is significantly different from that of the mock methylated construct (p < 0.05).
IV.4.2.2 The effects of DNA methylation and methylation density on Sp1-mediated eNOS promoter activity in Drosophila cells (46).

*Drosophila melanogaster* belongs to the group of eukaryotic organisms that lack methylation in its genome (245). Since *Drosophila* genomic DNA has been shown to be unmethylated, methylation-mediated effects on heterologous promoter/reporter constructs can be examined without any endogenous interference. Another advantage of this artificial system is that *Drosophila* does not express Sp1 or related proteins (63, 117, 245), as this will allow the further dissection of the role of Sp1/Sp3 and their possible interactions with methylated DNA. Since one of the CpG dinucleotides in the eNOS 5'-flanking region is situated in the middle of a functionally important high affinity Sp1 site (PRD 1), it is a logical extension to determine whether methylation of CpG doublets will affect the Sp1-dependent transcriptional activity.

To study the effects of methylation and methylation density on eNOS promoter activity driven by transcription factor Sp1, *in vitro* methylated promoter/reporter constructs by *HpaII* and *SssI* methylases, were transiently transfected into the *Drosophila* Schneider SL2 cells along with half-maximal amounts of pPacUSp1 (40 ng). In the absence of Sp1, the pGL2-1193/+109 promoter/reporter construct showed functional activity equivalent to mock-transfected cells. Results showed that high-density methylation decreased Sp1-driven eNOS promoter activity by approximately 45% compared to mock and untreated constructs (Figure 39). The effects of low-density methylation by *SssI* and *HpaII* are similar in that they only induced a 20% drop in promoter activity compared to mock and untreated. It is known that Sp1-binding is not affected
Figure 39. The effects of DNA methylation and methylation density on Sp1-mediated eNOS promoter activity in *Drosophila* Schneider SL2 cells. To study the effects of methylation and methylation density on eNOS promoter activity directed by Sp1, *in vitro* methylated promoter/reporter constructs (by *SssI* and *HpaII* methylases) were transiently transfected into SL2 cells along with 40 ng pPacUSp1. The data represent the mean ± S.E.M. Shown is a representative experiment (triplicate determinations), n=3. High methylation density exhibited a 40% reduction in promoter activity, while other low-density methylation constructs showed no significant difference compared to the mock-methylated construct. * indicates that the promoter activity is significantly different from that of the mock methylated construct (p < 0.05).
by methylation (35, 129, 266); however, these results clearly indicate that methylation alters Sp1 transactivation. This may suggest that methylation interferes with Sp1 transactivation via an indirect mechanism, perhaps through the condensation of chromatin or recruitment of other proteins into the vicinity or altering protein-protein interactions rather than directly inhibiting the binding of Sp1.

To further define the molecular basis of methylation-mediated repression on Sp1 transactivation, a further series of transient transfection experiments were performed in Drosophila SL2 cells. In vitro methylated (SssI high-density methylation and SssI low-density methylation) and mock-methylated pGL2-1193/+109 promoter/reporter constructs were co-transfected with increasing levels of a heterologous eukaryotic expression vector encoding Sp1 (pPacUSp1) (Figure 40) (n=3, triplicate determinants). Adding increasing amounts of Sp1 resulted in a concentration-dependent increase in functional promoter activity. The dose-response curves demonstrated that 0.2 and 10 ng Sp1 barely transactivated the promoter/reporter constructs. 40 ng of Sp1 yielded half-maximal activation while 100 ng gave even higher activity. The trend of transactivation by Sp1 appears to be the same in all three constructs with the exception that the SssI high-density and SssI low-density methylation constructs yielded proportionately lowered activity at each of the concentrations. Moreover, the SssI high-density methylation construct had a greater effect than the SssI low-density methylation construct. It is interesting that the curves were not shifted to the right or to the left, instead, for each amount of Sp1, activity was blunted proportionally.

Since DNA methylation does not affect Sp1 binding, it must disrupt Sp1 transactivation via an indirect mechanism. Although the mechanisms through which methylation acts on Sp1-mediated activation of the eNOS promoter remain to be elucidated, methylation density is clearly an important determinant.
Figure 40. The effects of DNA methylation and methylation density on the dose-dependent Sp1 activation eNOS promoter activity in *Drosophila Schneider SL2* cells. *In vitro* methylated promoter/reporter constructs (high- and low-density methylation by SssI methylase) were transiently transfected into *Drosophila* SL2 cells along with increasing amounts of pPacUSp1. The data represent the mean ± S.E.M. (n=3, triplicate determinations). Sp1 activation is dose-dependent and methylation decreases eNOS promoter activity at every concentration of Sp1 added.
Both DNA methylation and methylation density affect the synergistic activation of eNOS promoter by Sp1, Sp3, and Ets-1 in Drosophila cells (46).

We previously proposed a model in which Sp1, Sp3, and Ets-1 are essential trans-factors necessary for functional promoter activity of the human eNOS gene (Chapters III) (156). Mutating any of the activator recognition sites by these factors found in PRD I and II resulted in a marked decrease in functional promoter activity. In order to examine the effects of methylation and methylation density on the synergistic activation of the eNOS promoter by trans-factors Sp1, Sp3, and Ets-1, in vitro methylated promoter/reporter constructs were transiently transfected into Drosophila Schneider SL2 cells along with threshold amounts (10 ng) of each of Sp1 (pPacUSp1), Sp3 (pPacUSp3), and Ets-1 (pPacUEts-1) expression vectors (n=3, triplicate determinants). As a whole, the effects of methylation on Sp1-mediated eNOS transactivation were modest compared to the effects of methylation on the synergistic activation elicited by the combined addition of threshold amounts of Sp1, Sp3, and Ets-1. Results demonstrated that methylation at a high-density had a dramatic effect in that there was a significant decrease in promoter activity compared to the mock-methylated constructs (Figure 41). The SssI high-density methylation construct exhibited less than 10% of the mock-methylated promoter activity. Methylation at lower densities (SssI low-density methylation and HpaII methylation) did not affect the synergistic transactivation of the three factors to the same extent as high-density methylation. The SssI low-density methylation and HpaII-methylation constructs demonstrated approximately 30% and 45% promoter activity, respectively, compared to the mock-methylated construct. Taken together, these results suggest that repression of the synergistic action of Sp1, Sp3, and Ets-1 may be a function of methylation density.
Figure 41. The effects of DNA methylation and methylation density on the synergistic activation of eNOS promoter by Sp1, Sp3, and Ets-1 in *Drosophila* Schneider SL2 cells. To examine the effects of methylation and methylation density on eNOS promoter activity directed by Sp1, Sp3, and Ets-1, *in vitro* methylated promoter/reporter constructs (by SssI and HpaII methylases) were transiently transfected into *Drosophila* Schneider SL2 cells along with 10 ng pPacUSp1, pPacUSp3, pPacUEts-1. The data represent the mean ± S.E.M. Shown is a representative experiment (triplicate determinations), n=3. * indicates that the promoter activity is significantly different from that of the mock methylated construct (p < 0.05). High methylation density induced a 95% decrease in promoter activity, while other low-density methylation constructs showed less significant reduction (65% and 50% drop) compared to the mock-methylated construct.
IV.4.2.4 MeCP-2 further represses methylation-mediated transcriptional repression (46).

In addition to its direct effect on trans-factor binding, possible indirect mechanisms through which methylation represses transcription include the assembly of methylated templates into inactive chromatin formation and the binding of methyl-CpG-binding proteins. Since Drosophila genomic DNA is not methylated (245), Schneider SL2 cells presumably do not contain methyl-CpG-binding proteins. Therefore, any methylation-induced repression of the eNOS promoter activity seen in Drosophila Schneider SL2 cells cannot be due to interference by methyl-CpG-binding proteins, rather, it may be attributed to an indirect effect of chromatin assembly. To further dissect the role of methyl-CpG-binding proteins in methylation-mediated transcription repression, a heterologous eukaryotic expression vector encoding MeCP-2 (pPacUMeCP-2) was constructed for transient expression in the Drosophila Schneider SL2 system, which is devoid of endogenous MeCP-2. The in vitro methylated SssI high-density methylation and SssI low-density methylation pGL2-1193/+109 promoter/reporter constructs were co-transfected with half-maximal dosage of pPacUSp1 (40 ng) and increasing levels of pPacUMeCP-2 (Figure 42) (n=3, triplicate determinants). In the absence of MeCP-2, the SssI high and SssI low constructs exhibited only lowered promoter activity compared to the mock-methylated construct. Adding increasing amounts of MeCP-2 expression vector resulted in a concentration-dependent reduction in functional promoter activity for all three constructs (mock-methylated, SssI high-density methylation, SssI low-density methylation). Maximum repression of all three constructs occurred at 250 ng MeCP-2. The values of each dose-dependent curve are normalized to the value of 0 ng MeCP-2 as the baseline. This comparison shows that the half-maximal amount of MeCP-2 necessary for repression the mock-methylated construct is 5-fold.
Figure 42. MeCP-2 further represses methylation-mediated transcriptional repression of Sp1-directed eNOS promoter activity in Drosophila Schneider SL2 cells. In vitro methylated promoter/reporter constructs were transiently transfected into Drosophila Schneider SL2 cells with 40ng of pPacUSp1 and various amounts of MeCP-2. The data represent the mean ± S.E.M. Shown is a representative experiment (triplicate determinations), n=3. The values of each dose-dependent curve is normalized to the value of 0 ng MeCP-2 as the baseline. This comparison shows that the half-maximal amount of MeCP-2 for repression for the mock-methylated construct is 5-fold and 10-fold greater than that of the SssI low-density methylation and the SssI high-density methylation constructs, respectively, indicating that the effect of MeCP-2 is methylation density dependent.
and 10-fold greater than that of the SssI low-density methylation and the SssI high-density methylation constructs, respectively. These results indicate that the effect of MeCP-2 is methylation density-dependent. A surprising finding was the MeCP-2-mediated repression observed in the promoter activity of the mock-methylated construct. The mock-methylated construct underwent the same methylation and purification reactions as the other methylated constructs; however, no methylase was added in the initial methylation reaction. The non-methylated status of the mock-methylated construct was further confirmed by MspI and HpaII restriction digests. Nan et al. recently reported that MeCP-2 possesses an active transcriptional repression domain capable of long-range repression in vivo (222). This transcriptional repression domain may be responsible for the anomalous effect of MeCP-2 on the mock-methylated construct and will be discussed further.

To assess the effect of MeCP-2 on the methylation-mediated repression of Sp1, Sp3, and Ets-1 synergistic activation of the eNOS promoter, a series of in vitro methylated constructs (SssI high-density methylation, SssI low-density methylation, and HpaII-methylation) were co-transfected with 10 ng of each of pPacUSp1, pPacUSp3, pPacUEts-l and 100 ng pPacU MeCP-2 (Figure 43) (n=3, triplicate determinants). Results demonstrated that MeCP-2 further repressed the reduction in eNOS promoter activity induced by methylation. The addition of 100 ng of MeCP-2 further repressed the already minimal promoter activity of the SssI high-methylation construct, abolishing the residual activity, while the promoter activity of the SssI low-methylation construct was reduced by approximately half. The substantial reduction in promoter activity was seen in the HpaII-methylation construct where activity incurred a five-fold decrease, suggesting that the binding of MeCP-2 to certain methylated HpaII site(s) severely affects the ability of Sp1, Sp3, and Ets-1 to transactivate the eNOS promoter synergistically.
Figure 43. MeCP-2 further represses methylation-mediated transcriptional repression of the synergistic activation of eNOS promoter by Sp1, Sp3, and Ets-1 in Drosophila Schneider SL2 cells. *In vitro* methylated promoter/reporter constructs were transiently transfected into *Drosophila* Schneider SL2 cells with 10 ng each of the trans-factors and 100 ng MeCP-2. The data represent the mean ± S.E.M. Shown is a representative experiment (triplicate determinations), n=3. * indicates that the promoter activity is significantly different from that of the mock methylated construct (p < 0.05). † indicates that the promoter activity after the addition of MeCP-2 is significantly different from that without MeCP-2 (p < 0.05). The most drastic effect is observed in the *HpaII*-methylation construct where the addition of MeCP-2 decreased the residual activity by 5-fold compared to the 2-fold decrease seen in the *SssI* low-density methylation construct. Adding MeCP-2 abolishes the already low activity of the *SssI* high-density methylation.
This would indicate that certain *HpaII* site(s) is (are) situated at a critical region in the eNOS promoter. An obvious candidate is the *HpaII* site within PRD II where the three *trans*-factors are proposed to form multiprotein complexes (Chapter III) (156).

**IV.4.2.5 Methylation-mediated repression of eNOS promoter activity can be relieved by an excess of non-specific, methylated mimic DNA (46).**

Methylation-mediated transcriptional repression by histone H1 (181) or methyl-CpG-binding proteins (34, 180) has been shown to be reversed by co-transfection with methylated promoterless DNA. To study the effects of methylated competitor DNA, co-transfection experiments were performed in BAEC in which *in vitro* methylated pGL2-1193/+109 (*SssI* high-density methylation, *SssI* low-density methylation, and *HpaII*-methylation) was co-transfected with excess unmethylated or methylated plasmid DNA (pBluescript SK(-)) (Figure 44) (n=3, triplicate determinants). Co-transfection of excess methylated mimic DNA partially restored the activity of both the *SssI* low-density methylation and *HpaII*-methylation constructs; however, it was not able to rescue promoter activity when the construct was densely methylated as in the case of *SssI* high-density methylation construct. Similar rescue co-transfections in which methylation density affected the restoration of promoter activity have been previously reported in unrelated genes (34, 266).
Figure 44. Methylation-mediated repression of eNOS promoter activity can be relieved by an excess of non-specific, methylated mimic DNA. Co-transfection experiments were performed in BAEC in which in vitro methylated promoter/reporter constructs (SssI high-density, SssI low-density, and HpaII-methylation) were co-transfected with excess methylated or mock-methylated plasmid DNA (pBluescript SK(-)). The data represent the mean ± S.E.M. Shown is a representative experiment (triplicate determinations), n=3. * indicates that the promoter activity is significantly different from that of the mock methylated construct (p < 0.05). † indicates that the promoter activity after the addition of excess methylated plasmid DNA is significantly different from that with the mock methylated plasmid DNA (p < 0.05). Results indicate that excess methylated mimic DNA partially restored the activity of both the SssI low-density methylation and HpaII-methylation constructs, but not the SssI high-density methylated one.
IV.4.3  5-AZACYTIDINE-INDUCED HYPOMETHYLATION STUDIES

IV.4.3.1 Effects of DNA demethylation on eNOS gene expression in human cell lines (46).

The preceding studies established a possible association between hypomethylation of the eNOS promoter and increased level of gene expression and sought to define the molecular mechanisms implicated. Hypomethylation of the eNOS gene was induced by 5-azacytidine in endothelial (HUVEC) and non-endothelial cells (HeLa, HepG2, JEG-3, and TGW) \((n=3)\). Cells were treated with 5 \(\mu\)M of this potent DNA methyltransferase inhibitor for seven days. Cells were split at routine seeding density and fresh 5-azacytidine containing medium was added every 48 hours. Total cellular RNA was extracted on day 7. Steady-state levels of eNOS mRNA transcripts were assessed using RNase protection assays (Figure 45). Undigested probe alone is 340 nucleotides in length and the expected size of the protected fragment size is 292 nucleotides. The eNOS steady state mRNA level was extremely high in the HUVEC samples as indicated by the strong signals in those lanes. The signal intensity increased slightly in the treated HUVEC sample compared to the untreated one. As expected, no signal was evident in the HeLa, JEG-3, and TGW samples in the absence of 5-azacytidine. However, a protected fragment was detected in these three cell lines following 5-azacytidine addition. The signal intensity also increased in HepG2 following demethylation compared with that of the untreated lane. These results demonstrated that eNOS mRNA steady state levels in all the non-endothelial cell lines increased, some from previously undetectable levels, following inhibition of DNA methyltransferase activity.
Figure 45. 5-azacytidine induced eNOS gene expression. To establish a possible association between DNA hypomethylation of the human eNOS gene and increased level of gene expression, 5-azacytidine was used to induce hypomethylation in endothelial (HUVEC) and non-endothelial (HeLa, HepG2 and JEG-3) cells. RNase protection assays were used to quantify total cellular steady-state RNA. Lanes 1 and 2 represent 10 mg of yeast RNA hybridized to the probe, digested or not digested with RNase, respectively. Lane 3 is molecular weight size standard. Lane 4 represents undigested probe alone (340 nt). (-) indicates the control sample where no 5-azacytidine was added; (+) indicates the treated sample where 5 mM of 5-azacytidine was added every 48 hours for 7 days. Results demonstrated an induction of eNOS gene expression in the non-endothelial cells.
IV.4 DISCUSSION

Using the sodium bisulfite genomic sequencing method we convincingly verified that the nine CpG dinucleotides in the eNOS proximal promoter region were completely unmethylated in HUVEC and HuMVEC (Figure 36 and 37). This is in stark contrast with the almost fully methylated region in TGW, peripheral blood leukocytes and HuVSMC. The nine CpG dinucleotides in JEG-3 were almost fully methylated; however, the CpG at -209 displayed an unusually low methylation density. Given that both cultured cell lines and freshly isolated human cells demonstrated comparable findings, these differences do not represent artifacts of established cell lines.

In HeLa and HepG2 genomic DNA, there seems to be a region of especially low methylation density around nt -100 relative to the transcription start site, which represents the high affinity Sp1 site of PRD I. It has been found that Sp1 helps maintain regions of DNA methylation-free (35, 196, 340). Moreover, the adjacent sites were found to be methylated to a much higher extent. Specifically, in HepG2, the -95 and -100 sites were only 10% methylated; however, merely 40 bp downstream is the -51 site which was found to be 60% methylated while the -167 site was found to be 90% methylated. Also, the -95 and -100 sites in HeLa were 20 and 10% methylated, respectively, but the -137 site, which is only some 40 bp away, was found to be 80% methylated and the downstream -51 site was found to be 30% methylated. This regional hypomethylation in these two cell types suggests that Sp1 may be binding to this high affinity site and subsequently preventing methylation or inducing methylation of this particular region.
Taken together as a whole, these data indicate that there is a significant difference between the methylation status of the eNOS proximal promoter in endothelial cells (HUVEC and HuMVEC) and that of the non-endothelial cell types examined. Furthermore, endogenous eNOS gene expression was induced in four non-endothelial cell types (HeLa, HepG2, JEG-3, and TGW) following demethylation by DNA-methyltransferase inhibitor, 5-azacytidine (Figure 45). Similar findings were found with methylation-sensitive isoschizomer mapping (data not shown). The methylation status of a larger region of eNOS genomic sequence (from −1193 to +1207) differed between the endothelial and non-endothelial cell types. In summary, these results demonstrate that the state of DNA hypomethylation is associated with eNOS gene expression.

Transient transfections of in vitro methylated promoter/reporter constructs (pGL2-1193/+109) in endothelial cells were performed to assess the functional consequences of CpG methylation in the human eNOS 5′-flanking region (Figure 38). These results evidently suggest a role for DNA methylation in the transcriptional repression of the human eNOS gene. Methylation density, the location of the methylated CpG dinucleotides, and the functional specificity of methylated cytosines in the presence of the CpG dinucleotides may be important determinants as demonstrated by the differences between the functional consequences of SssI high, SssI low, HpaII, and MspI methylase activity on functional promoter readouts in BAEC.

The repressive effects of methylation seen in the endothelial cell transfections may be attributed to the interactions of a number of different processes. To further study the functional correlation between DNA methylation and the gene activity of eNOS, an artificial system that lacks endogenous methylation is ideal. The Drosophila Schneider SL2 cell line was used to examine the effects of DNA methylation and methylation density on Spl-mediated eNOS promoter activity.
as well as the combined action of Sp1, Sp3, and Ets-1. Furthermore, the elucidation of the molecular mechanisms through which DNA methylation interferes with gene expression were made possible through these studies. The results of these studies showed that methylation clearly affected the ability of these DNA binding proteins to transactivate the eNOS promoter. The Sp1-mediated promoter activity decreased by approximately 45% when the promoter/reporter construct was highly methylated (Figure 39). Since Sp1-binding has been shown to be unaffected by CpG methylation (35, 129, 266), the mechanism of repression is likely to be an indirect one, possibly involving methylation-mediated condensation of the chromatin or the recruitment of methyl-CpG binding proteins.

_Drosophila_ genomic DNA has been shown to be unmethylated (245), hence cells from such organisms presumably do not contain methyl-CpG-binding proteins. This suggests that the mechanism of repression seen in these cells may be independent of these proteins, and that methylation is promoting the preferential condensation of methylated chromatin into a repressive higher order structure. Several lines of evidence have been cited in the literature to support the view that methylation modulates higher order chromatin structure: (i) methylated thymidine kinase gene promoter activity is inhibited only after the DNA has been assembled into chromatin (39). (ii) methylated DNA is preferentially located in nucleosomes containing histone H1 (15). (iii) histone H1 has been shown to inhibit transcription from methylated templates (181), and (iv) transfected methylated DNA is more resistant to endonucleases than unmethylated DNA (161).

As a whole, the effects of DNA methylation on Sp1-mediated eNOS transactivation (Figure 39) were modest compared to that on the synergistic activation of the combined addition of Sp1,
Sp3, and Ets-1 (Figure 41). The extreme sensitivity of the Sp1/Sp3/Ets-1 synergy to methylation-induced repression may be due to the requirement for precise stereo-specificity for the formation of a highly coordinated complex, which involves DNA activator regions, as well as multiprotein complexes. Moreover, methylation of the CpG dinucleotide at the HpaII/MSpI site within PRD II may be critical because it can result in the alteration of the physical properties of the DNA helix, or the recruitment of methyl-binding proteins such as MeCP-2 that may block the recognition sites for the other functionally essential trans-factors and further hinder nucleoprotein formation.

To further dissect the indirect mechanisms that are responsible for the methylation-mediated transcriptional repression of the eNOS gene and the possible role of methyl-CpG-binding proteins, in vitro methylated pGL2-1193/+109 promoter/reporter constructs were co-transfected with Sp1 alone or Sp1, Sp3, and Ets-1, along with MeCP-2 into Drosophila Schneider SL2 cells. Results showed that the addition of MeCP-2 further represses methylation-mediated repression of Sp1-directed and Sp1/Sp3/Ets-1-mediated eNOS activity in a concentration- as well as methylation density-dependent manner (Figure 42, 43). MeCP-2 had an especially prominent effect on methylated constructs in the presence of Sp1 alone.

Methylation of non-chromatin templates alone affected the synergistic transactivation of the eNOS promoter by Sp1, Sp3, and Ets-1 in SL2 cells (Figure 41). The addition of MeCP-2 was demonstrated to further repress functional promoter activity mediated by the combined action of these trans-factors (Figure 43). MeCP-2 reduced the minimal promoter activity of the SssI high construct to background activity and it induced a 50% reduction in the SssI low construct activity. The strongest effect was seen in the HpaII construct where the activity decreased five-
fold. This suggests that the binding of MeCP-2 to certain methylated *HpaII* site(s) interferes with the ability of Sp1, Sp3, and Ets-1 to transactivate the eNOS promoter synergistically, indicating that the site(s) of the *HpaII* sequence is (are) located in a critical region in the eNOS promoter. Sequence inspection revealed a *HpaII* site within PRD II between the Sp1/Sp3 and the Ets-like binding sites, the domain where the proposed multi-protein complex of Sp1, Sp3, and Ets-1 form. This finding reiterates the importance of critical positioning of methylated CpG dinucleotides within the promoter region.

An intriguing observation was the MeCP-2-mediated repression seen in the promoter activity of the mock-methylated construct in *Drosophila* Schneider cells. The non-methylated status of the construct was confirmed by methylation-sensitive isoschizomer digestions and was not explained by endogenous bacterial methylase effects on reporter constructs. We suspect that the active transcriptional repression domain of MeCP-2 may be responsible for the anomalous effect on the mock-methylated construct. Although this mechanism of repression is not fully understood, it has been suggested that this repressive effect may be exerted through protein-protein interactions. An exciting new finding was recently reported by both Jones et al. and Nan et al. Transcriptional repression by MeCP-2 is achieved, in part, through its recruitment of a histone deacetylase complex (150, 223). This novel finding bridges two global mechanisms of gene regulation, DNA methylation and histone deacetylation, by MeCP-2. It is intriguing that *Drosophila* Schneider cells, though deficient in endogenous methylase activity and functional methyl-binding proteins, exhibit acetyltransferase and deacetylase activity. One plausible explanation for the repression of the mock-methylated construct is that MeCP-2 may not be interacting directly with the DNA; rather, it may be involved in the formation of multi-protein
complexes involving histone deacetylases and transcriptional co-repressors which bind directly to the DNA.

Moreover, in support of an indirect mechanism model, we also found that the methylation-mediated repression of the eNOS promoter can be partially relieved by co-transfection with non-specific, methylated competitor DNA (Figure 44). Excess non-specific, methylated competitor DNA has previously been shown to reverse the methylation-induced repression by methyl-CpG-binding proteins (34, 180) and histone H1 (181). Thus, DNA methylation appears to repress the eNOS promoter through an indirect mechanism. Furthermore, successful transfection of excess methylated DNA indicates that DNA methylation did not affect transfection efficiency.

Taken together, our results suggest that DNA methylation plays an important role in both the transcriptional regulation and endothelial cell-specific expression of the human eNOS gene. The unmethylated status of the proximal promoter region of eNOS correlates with gene activity in eNOS-expressing HUVEC. Functional expression studies revealed that the methylation-mediated transcriptional repression involves both direct and indirect mechanisms. Both the assembly of methylated DNA into an inactive chromatin structure and binding of methyl-CpG-binding proteins, specifically MeCP-2, play vital roles in this repression. In addition, the disruption of enhanceosome formation either through the condensation of chromatin or by the binding of MeCP-2 may also contribute to transcriptional inactivation.

We posit that the eNOS promoter contains specific methylation control regions responsible for demethylation of the promoter in an endothelial cell-specific fashion. Our in vivo model supports this hypothesis. A promoter/reporter insertional transgene containing the native murine
eNOS promoter (-5200/+28) directed transcription of nuclear-localized β-galactosidase in an endothelial cell-specific fashion. This in vivo expression profile was uniform across multiple founders and not dependent upon the site of integration into mouse genomic DNA. The findings of this work demonstrated that murine eNOS genomic regions spanning -5200/+28, relative to the transcription start site, directed expression of a reporter construct in a fashion that recapitulated the known expression profile of eNOS mRNA and protein. Thus this murine promoter/reporter construct contains requisite cis-elements necessary for control of methylation and chromatin opening that allow efficient β-galactosidase expression, mimicking the eNOS expression profile, by virtue of the presence of the demethylation control region. Future studies will address the mechanism of regulation of the demethylation control region. Specifically, we will evaluate the methylation status of the native murine eNOS promoter and compare this with the methylation status of three independent insertional transgene murine eNOS promoter/nls-βgal mouse lines. In this regard we are especially interested in the GATA site at -230. GATA member families have been demonstrated to play an important role in LCR (locus control region) structure and function (244, 250). It would be especially interesting if GATA facilitates the correct structural organization of the eNOS promoter thus permitting robust expression. The putative GATA cis-DNA element is conserved across species (please see next chapter).

The described studies are relevant to our understanding of gene regulation in endothelial cells, as well as the regulation of gene expression in the cardiovascular system. eNOS is the first constitutively expressed gene in the vascular endothelium whose expression has been shown to be regulated by DNA methylation.
CHAPTER V

CHARACTERIZATION OF THE HUMAN eNOS PROMOTER

(PART III)

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in the human eNOS gene. IN PREPARATION (1999)
CHAPTER V. CHARACTERIZATION OF THE HUMAN eNOS PROMOTER
(PART III)

V.1 OBJECTIVE AND AIMS

Sequence comparison of the eNOS promoter across species (human, murine and bovine) reveals a high degree of sequence conservation with respect to trans-factor binding sites (Figure 47). Specifically, the Sp1/Sp3 sites found in PRD I and II are conserved across species. The high degree of relatedness of PRD I and II across species supports the importance and relevance of these functionally important domains in the transcriptional control of the eNOS promoter.

Sequence inspection of regions downstream of PRD I evidenced certain regions with a high degree of sequence identity. Specifically, an E box (5'- CANNTG -3'), a GATA site (5'- WGATAR -3') and an Ets site are conserved upstream of PRD I and II (Figure 47). In addition, two palindromic Ets sites at approximately -37 and -30 relative to the human major start site of transcription are present in the human, murine and bovine eNOS promoters. As described in Chapter 1, the human eNOS promoter does not contain consensus TATA or Inr elements, yet 80% of mRNA transcripts initiate at a unique site (Figure 1A). Thus the possibility exists that a novel regulatory mechanism is implicated in transcription initiation for the eNOS gene in vascular endothelial cells. Curiously, the palindromic Ets elements are critically positioned at helix sites normally occupied by the TATAA box in other genes. Therefore, the role of the conserved palindromic Ets sites in the human eNOS gene will be explored.
V.1.1 OBJECTIVE

The overall objective of these studies is to examine the basic regulatory features implicated in the physiologic and pathophysiologic control of human eNOS gene transcription in vascular endothelial cells.

V.1.2 SPECIFIC AIMS

1. The first specific aim of this chapter is to define the functionally important \textit{cis}-DNA elements in the human eNOS 'minimal core' promoter 3' of -45, using linker-scanning mutagenesis of promoter / reporter constructs.

2. The second specific aim of these studies is to identify the functionally important \textit{cis}-regulatory elements and the biologically important \textit{trans}-acting factors that account for Positive Regulatory Domain III in vascular endothelial cells.

3. The third specific aim of these studies is to characterize the functional importance of the above \textit{cis}-DNA elements and corresponding \textit{trans}-acting factors in the regulation of eNOS promoter activity in a heterologous \textit{Drosophila} expression system.

V.2 HYPOTHESIS

Positive Regulatory Domain III, which spans a 20 bp sequence from -44 to -25, represents an important functional domain in the constitutive expression of eNOS in vascular endothelial cells. Functional contributions are expected from Ets family members, Sp1 and Sp3 \textit{trans}-factors.
V.3 MATERIALS AND METHODS

Materials. Materials are as described in Chapter III.

Cell Culture. Bovine aortic endothelial cells (BAEC) and Schneider Drosophila cells (SL2) were propagated as described in Chapter III.

-1193/+109 linker mutants. Five linker-scanning mutations were created that span a 50 base pair region of the human eNOS promoter from -44 to +16, relative to the start site of transcription (Figure 46). These mutations were incorporated into the pGL2 -1193/+109 construct. The pGL2 -1193/+109 construct was created as previously described (Chapter II) (9). The primers used to generate these constructs are listed in Table VI. To confirm the incorporation of the final 10 bp linker mutation in the pGL2 -1193/+109 promoter / luciferase construct, the positive clones were once again sequenced using the automated ABI Prism 377 DNA sequencer. In turn, each positive clone was purified using a CsCl "double banding" plasmid purification procedure to provide transfection-grade DNA.

Transient Transfection Assays. All transient transfections were carried out using the Lipofectin Reagent (Gibco BRL), as described in Chapter II. pCGN-MAZ and pCGN (empty vector) was kindly provided by Thomas Shenk (Princeton, NJ) (242).

Protein Extraction, Luciferase Assays, β-Galactosidase Assays, and Measurement of Protein Concentration. All were performed as described in Chapter II.
Figure 46. Schematic representation of the proximal promoter of the human eNOS gene and new linker-scanning mutations. PRD I and II are indicated. Positions of new linker-scanning mutations are indicated.
**Primers and Oligonucleotides.** All oligonucleotides were created using the Beckman Oligo 1000 DNA synthesizer (Beckman Instruments, Mississauga, Ontario). The primers used to create the -1193/+109 linker mutants are listed in Table VI. Oligonucleotides used in EMSA analysis are listed in Table VII.

**Preparation of Nuclear Lysates.** Nuclear lysates were collected as described by (279) and in Chapter II.

**Electrophoretic Mobility Shift Assays (EMSA).** Single-stranded oligonucleotides were synthesized using the Beckman Oligo 1000 DNA synthesizer (Table VII). EMSA analysis was performed as described in Chapter II.

Where appropriate, non-labeled ('cold'), competitive oligonucleotides were added 10 minutes before addition of labeled probe. In reactions where recombinant Ets proteins were used in EMSA, 0.03 % NP40 was added to the binding reaction and 0.05 % NP40 was added to the acrylamide gel. For “supershift” analyses, antibodies were added 30 minutes after probe addition and incubated a further 20 minutes at 22 °C. Monoclonal and polyclonal antibodies were from Santa Cruz Biotechnology: Sp1 (PEP2), Sp2 (K-20), Sp3 (D-20), Sp4 (K-20), Ets-1 (NH₂-terminus, N-276), Ets-1 (COOH-terminus, C-20), Ets-2 (C-20), PU.1 (Spi-1, T-21), Erg-1 (C-17), Fli-1 (C-19), PEA3 (16) and Elk-1 (I-20). Anti-Elf-1 (rabbit polyclonal) was a generous gift from J. M. Leiden (Chicago, IL) (179).

**In vitro DNase I footprinting analysis.** The pGL2-1193/+109 construct was used for the footprinting experiment. Fourteen μg of HUVEC or HeLa nuclear extract was incubated with 24
Table VI. Primer pairs used to construct the new pGL2-1193/+109 linker-scanning mutants.

<table>
<thead>
<tr>
<th>NAME</th>
<th>PRIMER SEQUENCE</th>
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<tbody>
<tr>
<td>A main</td>
<td>5' - CCT TTA TGA CCC CCT GGT G -3'</td>
</tr>
<tr>
<td>B main</td>
<td>5' - TTT ATG TTT TGT GCG TCT TGC -3'</td>
</tr>
<tr>
<td>A 44-15</td>
<td>5' - CGA GAT CTG CGG GAC CGA GAG GAG G -3'</td>
</tr>
<tr>
<td>B 44-15</td>
<td>5' - CGG GAT CCG CCT AAG GAA AAG GCC A -3'</td>
</tr>
<tr>
<td>A 144-15</td>
<td>5' - CGA GAT CTG CGA AGA GGG AGG GGA C -3'</td>
</tr>
<tr>
<td>B 144-15</td>
<td>5' - CGG GAT CCG CGG CCA GGG CTC TGC T -3'</td>
</tr>
<tr>
<td>A 240-15</td>
<td>5' - CGA GAT CTG CTT TTC CTT AGG AAG A -3'</td>
</tr>
<tr>
<td>B 240-15</td>
<td>5' - CGG GAT CCG CCT GCT GGA GCA GCC A -3'</td>
</tr>
<tr>
<td>A 340-15</td>
<td>5' - CGA GAT CTG TAG CCC TGG CCT TTT C -3'</td>
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<tr>
<td>B 340-15</td>
<td>5' - CGG GAT CCG CAG GCA GCA GAG TGG A -3'</td>
</tr>
<tr>
<td>A 44-16</td>
<td>5' - CGA GAT CTG CGG TTC AGC AGA GCC C -3'</td>
</tr>
<tr>
<td>B 44-16</td>
<td>5' - CGG GAT CCT AGT GGA CGC ACA GTA A -3'</td>
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<tr>
<td>A 144-16</td>
<td>5' - CGA GAT CTG CTC TGC TGC CTG CTC C -3'</td>
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<tr>
<td>B 144-16</td>
<td>5' - CGG GAT CCG CAG TAA CAT GGG CAA C -3'</td>
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Table VII. Oligonucleotides used in EMSA analysis.

<table>
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<td>-140/111</td>
<td>TCC CAG CCG GGC TTT CCC TCA CAT TGT GTA T</td>
</tr>
<tr>
<td>-155/120</td>
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<td>CCC CTC CTC TCG TGT CCC TCC CTC TCC TTA AGG AA</td>
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<td>Fli-1-E4</td>
<td>GTG CCG GGG TAG GAA GTG GGC TGG G</td>
</tr>
<tr>
<td>-45/19</td>
<td>CCT CCC TCT TCC TAA GGA AAA GGC CAG</td>
</tr>
<tr>
<td>-45/19(mut 5' ↔ 3' Ets)</td>
<td>CCT CCC TCT TAA TAA TTA AAA GGC CAG</td>
</tr>
<tr>
<td>-45/19(mut 5' ↔ Ets)</td>
<td>CCT CCC TCT TAA TAA TTA AAA GGC CAG</td>
</tr>
<tr>
<td>-45/19(mut 3' → Ets)</td>
<td>CCT CCC TCT TCC TAA TTA AAA GGC CAG</td>
</tr>
<tr>
<td>-45/19(mut-dir-Ets (5' → 3' →))</td>
<td>CCT CCC TCG GAA TAA GGA AAA GGC CAG</td>
</tr>
<tr>
<td>HIV-2 LTR</td>
<td>TCG AGT TAA AGA CAG GAA CAG CTA TGT CGA</td>
</tr>
<tr>
<td>HTLV I-LTR</td>
<td>TCG AGG GGA GGA AAT GGG TGT CGA</td>
</tr>
<tr>
<td>PEA3</td>
<td>TCG AGC AGG AAG TGA CGT CCG</td>
</tr>
<tr>
<td>STROM</td>
<td>TCG AGC AGG AAG CAT TTC CTG GTC GC</td>
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*bold/underlined sequence refers to mutated nucleotides*
fmol of DNA in a 40 μl total volume binding reaction (49.5 mM Tris-HCl (pH 7.6), 20% glycerol, 100 mM KCl, 2.1 mM EDTA, 12.2 mM MgCl₂, 2 μg of poly d(I:C), 60 μg BSA). The binding reaction was incubated on ice for 20 minutes and 1 μl of 0.1 M CaCl₂ was added. DNase I digestion followed using titrated amounts of enzyme (1-100 ng) in order to cleave the DNA template at a limited number of sites, and incubating on ice for 2 minutes. To stop the reaction 41 μl of DNase I stop buffer was added (10 mM HEPES (pH 7.6), 20 mM EDTA, 1% SDS, 5 μg/ml yeast tRNA). The digested DNA was subsequently phenol:chloroform extracted and precipitated using a standard sodium acetate:ethanol precipitation. The precipitated DNA was then subjected to linear arithmetic PCR using 0.5 pmol of an antisense 5' end-labeled primer (EN-A+28, 5' - GGCCACGCTCTTCAAGTTG -3') or a sense primer (EN-S-235, 5' - CCACCTTATCAGCCTCAGT -3') using 2.5 Units of Taq polymerase. This allowed footprinting of the bottom and top strands respectively. PCR reaction involved a 5 minute hot start at 95 °C and 10 cycles of 1 min at 94 °C, 1 minute at 60 °C, 2 minutes at 72 °C. The labeled PCR product was extracted and precipitated and resuspended in 4 ml of loading buffer (80% formamide, 5 mM Tris-Borate (pH 8.3), 1 mM EDTA, 0.1% Xylene Cyanol, 0.1% Bromophenol Blue). The PCR product was heated at 90 °C for 5 minutes and run on a 6% denaturing sequencing gel. For the DNA size markers, the same labeled primer was used to produce a sequencing ladder using the SEQUENASE KIT.

**Nuclear run-off analysis.** Nuclear run-off analysis was performed as previously described (29). The human eNOS 5'-specific probe was a 1.3 kb *BamHI-SmaI* genomic fragment from the eNOS genomic clone MDN 30 spanning −530 through +780 nucleotides relative to the start site of transcription and includes exon 1 and 5' half of intron 1. The mid region probe was a 1.1 kb *EcoRI* cDNA fragment from plasmid PM7 spanning exons 8 through 16 and the 3' probe
consisted of a 1.6 kb \textit{KpnI} cDNA fragment from plasmid BCD 4 spanning exons 17 through 26. The human \textit{c-fos} probe was a 2.2 kb \textit{Scal-BamHI} genomic fragment from the plasmid pF711 spanning intron 2 through 1 kb downstream of the site of cleavage/polyadenylation. pBSK II (-) linearized using EcoRI was used as a negative control. A 547 bp HindIII-Xbal human GAPDH cDNA fragment was used from plasmid PCR II-HuGAPDH to normalize for inter-sample variability and as positive control.

\textbf{Data Analysis.} Unless otherwise indicated, data are expressed as the mean ± S.E.M. obtained in at least three independent experiments, each done in triplicate. When comparisons were made we used analysis of variance (ANOVA), followed by the Student-Newman-Keuls test. The level of statistically significant difference was defined as $p<0.05$. 

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V.4 RESULTS

V.4.1 SEQUENCE ALIGNMENT OF HUMAN eNOS MINIMAL CORE PROMOTER ACROSS SPECIES

Our laboratory cloned and characterized the murine eNOS promoter (303). Sequence comparison of the eNOS promoter across species (human, murine and bovine) revealed a high degree of sequence conservation with respect to trans-factor binding sites (Figure 47). Specifically, the Sp1/Sp3 sites found in PRD I and II are conserved across species. The Ets site and the YY1 site in PRD II are also conserved. The high degree of relatedness of PRD I and II across species supports the importance and relevance of these functionally important domains in the transcriptional control of the eNOS promoter. Sequence inspection of regions upstream of PRD II evidenced regions of conservation spanning an E box site (5' - CANNTG -3'), a GATA site (5' - WGATAR -3') and an Ets site (5' - GGAA -3'). Sequence inspection of regions downstream of PRD I evidenced certain regions with a high degree of sequence identity. Specifically, two palindromic Ets sites at approximately -37 and -30, relative to the human major start site of transcription, are present in the human, murine and bovine eNOS promoters. To address whether these elements play a role in the transcriptional regulation of the eNOS promoter, a further series of linker-scanning mutant constructs were generated.
Figure 47. Cross-species alignment of the murine, bovine and human eNOS proximal promoter regions. Enclosed sequences denote regions of nucleotide identity. Conserved transfactor binding sites and PRD I and II are illustrated. Human eNOS +1 site is illustrated.
V.4.2 TRANSIENT TRANSFECTION FUNCTIONAL STUDIES IN BAEC

V.4.2.1 Identification of a third Positive Regulatory Domain (PRD III) in the human eNOS core promoter

To define the functionally important cis-acting DNA elements in the human eNOS minimal core promoter, a systematic mutational analysis of the proximal promoter region of the eNOS gene was undertaken. Specifically, six new 10 bp linker-scanning mutant constructs were created in the context of the maximally active pGL2 -1193/+109 promoter / luciferase construct and spanned a 60 bp region from -44 to +16 relative to the start site of transcription. These mutants are site-specific with each representing the same 10 bp sequence [5' - GCA GAT CCG C -3'].

Promoter / reporter expression of each linker-scanning mutant construct was assayed following transient transfections of BAEC (n=5, triplicate determinations) (Figure 48).

-54/-45mut linker-scanning constructs did not demonstrate any significant change in luciferase activity, as compared to the wild type construct, pGL2 -1193/+109, as shown in Chapter II (Figure 11). On the other hand, -44/-35mut and -34/-25mut linker-scanning constructs displayed a significant 60-65% drop in functional promoter activity relative to the wild type construct (p<0.0001). Mutating the sequences between -24 and +6, as consecutive 10 bp mutations, resulted in no significant change in activity. On the other hand, the 10 bp mutation involving +7/+16 resulted in a 90% increase in luciferase activity, compared to the wild type promoter construct. -1193/+109 (p<0.0001), suggesting the presence of a negative regulatory element.
Figure 48. Activity profiles of human eNOS -1193/+109 promoter/reporter luciferase linker-scanning mutations in BAEC. Linker-scanning mutants introduced a 10 bp substitution [5'-GCAGATCCGC-3']. To control for transfection efficiency cells were co-transfected with pRSV-β-gal and relative luciferase activity was normalized for protein and β-galactosidase values. The data from these transient transfections are expressed as % luciferase activity relative to pGL2 -1193/+109 and represent the mean ± S.E.M. (one representative from 5 independent experiments, triplicate determinations). The maximally active construct, pGL2-1193/+109, displayed 10% to 20% of the activity an SV40 promoter/enhancer-directed luciferase control vector, pGL2-control.
Taken together, three of the eNOS promoter / reporter constructs from the linker-scanning mutant series resulted in a significant change in luciferase expression. These constructs defined a new positive regulatory domain, designated PRD III, from −44 to −24. The existence of PRD III was not initially suggested with deletion promoter / reporter constructs but only with the linker-scanning mutagenesis approach, specifically localizing it to a 20 bp of DNA sequence. Thus, this method may be used to further refine cis-regulatory DNA domains. Assuming no effects on reporter translation, the third linker-scanning mutation, +7/+16 identified a negative regulatory region present in the eNOS core promoter. This effect was consistent across varied lots of maxi-prep DNA and no effect on transfection efficiency was evident with this linker-scanning mutant construct. These results may indicate two things: i) that the 10 bp sequence from +7 to +16 contains a negative cis-DNA element that interferes with efficient transcription initiation and subsequent promoter activity, or ii) that the efficiency of translation of the luciferase reporter RNA was altered because the structure of the RNA was altered with the introduction of the 10 bp linker-scanning mutation. Future studies (eg. RNase protection and primer extension analysis) will be necessary to address the relative contributions of translation and/or transcription.

V.4.3 TRANS-ACTING FACTOR INTERACTION STUDIES USING EMSA ANALYSIS

V.4.3.1 Nucleoprotein complexes formed by PRD III

Sequence inspection of PRD III regions indicated a variety of putative DNA cis-elements: two palindromic Ets sites [GGAA/T] (168), low affinity Sp1 [5' - GGG AGG –3'] (14) and MAZ [GGGAGGG] (33, 74). In order to identify the trans-acting factors that interact with PRD III,
two oligonucleotides were designed spanning PRD III, -61/-27 and -45/-19 (Table VII). A 35-mer double-stranded DNA probe (-61/-27) representing residues -61/-27 in the human eNOS 5'-flanking region, and thereby spanning PRD III, formed a series of protein/DNA complexes with BAEC nuclear extracts (Figure 49, complexes A, B, C, D, E, F and G, lane 1). Upon exposure of these protein/DNA complexes to an antibody directed against Sp1, a supershift of complex A, the slowest migrating band, was observed (Figure 49, lane 2 and Figure 52, lane 3). With the addition of 100-fold molar excess of unlabeled -61/-27 probe, all complexes (A through F) were effectively competed away, with the exception of complex G, suggesting it is a non-specific complex (Figure 50, lane 3). Incubating protein/DNA complexes with polyclonal antibodies directed against Sp2 and Sp4 (data not shown), resulted in no supershift or shift abrogation. However, exposing protein/DNA complexes to a polyclonal antibody directed against Sp3 resulted in both supershift and shift abrogation of complexes B and C (Figure 51, lane 3). This is a similar trend demonstrated with EMSAs performed with oligonucleotides spanning PRD I (-120/-91) and the low-affinity Sp1 site in PRD II (-155/-120) (see Chapter III, Figures 15 and 17), suggesting that complex A contains a protein antigenically-related to Sp1 and complexes B and C contain proteins antigenically-related to Sp3. MAZ, a transcription factor that displays protean roles in transcription initiation, interference and termination (33), was shown to physically interact with PRD II in Chapter III. A monoclonal antibody directed against MAZ also resulted in shift abrogation of complexes formed upon the -61/-27 probe, especially the slower migrating complexes (complexes A through E, Figure 52, lane 4). This suggests that MAZ is capable of interacting with both PRD II and III.
Figure 49. EMSA of endothelial nuclear protein binding to -61/-27 PRD III eNOS 5'-flanking region and anti-Sp1. Lane 1 represents -61/-27 probe incubated with 10 µg of BAEC nuclear extract. In lane 2 anti-Sp1 was added to the binding reaction. 2.5 X10^4 dpm of labeled -61/-27 probe was used in the binding reaction. Arrows on left represent protein/DNA complexes and arrows on the right represent Sp1 supershifted complex.
Figure 50. Specific nucleoprotein complex formation with the -61/-27 PRD III eNOS 5'-flanking region. Lane 1 represents probe alone and in lane 2, 10 μg of BAEC nuclear extract was added. Seven protein/DNA complexes are evident: A, B, C, D, E, F and G. In lanes 3, 100-fold molar excess of -61/-27 was added to the binding reaction as competitors. 2.5 X10^4 dpm of labeled -61/-27 probe was used in the binding reaction. Arrows on left represent protein/DNA complexes.
Figure 51. EMSA of endothelial nuclear protein binding to -61/-27 PRD III eNOS 5'-flanking region and Sp1 family antibodies. Lane 1 represents -61/-27 probe incubated with 10 μg of BAEC nuclear extract. In lane 2 anti-Sp1 was added to the binding reaction. In lane 3 anti-Sp3 was added to the binding reaction. 2.5 X10^4 dpm of labeled -61/-27 probe was used in the binding reaction. Arrows on left represent protein/DNA complexes and arrows on the right represent Sp3 supershifted complex.
Figure 52. EMSA of endothelial nuclear protein binding to -61/-27 PRD III eNOS 5'-flanking region. Lane 1 represents -61/-27 probe alone. Lane 2 represents -61/-27 probe incubated with 9 µg of BAEC nuclear extract. In lane 3 anti-Sp1 was added to the binding reaction and in lane 4 anti-MAZ was added. 2.5 X10^4 dpm of labeled -61/-27 probe was used in the binding reaction. Arrows on left represent protein/DNA complexes and arrows on the right represent supershifted complexes.
A series of protein/DNA EMSA complexes were observed with the labeled –45/-19 probe and BAEC nuclear extracts (Figure 53, lane 2). Upon addition of 100-fold molar excess of cold probe, clear competition was evident for all protein/DNA complexes (Figure 53, lane 3 and Figure 55, lane 3). Exposure of complexes formed with the –45/-19 probe to a rabbit polyclonal antibody directed against Elf-1 resulted in a supershift and shift abrogation of the fastest migrating complex as well as a change in the migration pattern of complexes H and I (Figure 53, lane 4). These findings imply that the fast migrating complex (complex J) contains a protein that is antigenically-related to Elf-1. These findings are taken to indicate that Elf-1 is present in endothelial cell nuclear extracts and that this Ets family member can also participate in nucleoprotein complex formation with PRD III. Addition of monoclonal or polyclonal antibodies directed against varied members of the Ets member family were exposed to the nucleoprotein complexes formed with the –45/-19 probe. These antibodies have been demonstrated to exhibit cross-reactivity across species. Anti-Ets-1, anti-Elf-1, anti-Ets-2, anti-Erg-1, anti-PEA3 and anti-Elk-1 (data not shown) failed to modify the nucleoprotein complexes. However, upon the addition of anti-Pu.1 and anti-Fli-1, a supershift was evident (Figure 54, lanes 3 and 4, respectively). Clear competition of protein/DNA complexes H and I was evident upon exposure to 100-fold molar excess of the oligonucleotide spanning the 5′-end of PRD III, -61/-27 (Figure 55, lane 4). A similar reduction is also evident with the cold competitor containing the E4 Ets binding site from the Flt-1 promoter, Flt-1-E4 (Figure 55, lane 5). Sequence alignment of the Ets sites in PRD III with the Ets site found in the E4 element of the Flt-1 promoter revealed a high degree of sequence identity (Table VIII). The competition evidenced in lane 5 suggests that the Ets protein binding to the E4 site is also recognizing the Ets binding sites on –45/-19. Other Ets binding site-containing oligonucleotides, including the human T-cell lymphotrophic virus type I long terminal repeat (HTLV-I LTR) site (Ets-1) (106).
Figure 53. EMSA of endothelial nuclear protein binding to -45/-19 PRD III eNOS 5'-flanking region. Lane 1 represents -45/-19 probe alone. Lane 2 represents -45/-19 incubated with 10 µg of BAEC nuclear extract. In lane 3, 100X molar excess of -45/-19 competitor was incubated with the binding reaction. In lane 4, anti-Elf-1 was incubated with the binding reaction. 2.5 X10^4 dpm of labeled -45/-19 probe was used in the binding reaction. Arrows on left represent protein/DNA complexes.
Figure 54. EMSA of endothelial nuclear protein binding to -45/-19 PRD III eNOS 5'-flanking region and Ets antibodies. Lane 1 represents -45/-19 probe alone. Lane 2 represents -45/-19 incubated with 10 μg of BAEC nuclear extract. In lane 3 and 4, Ets antibodies were added to the binding reaction: PU.1 (lane 3) and Fli-1 (lane 9). 2.5 X10^4 dpm of labeled -45/-19 probe was used in the binding reaction. Arrows on left represent protein/DNA complexes and arrows on the right represent supershifted complexes.
Figure 55. EMSA of endothelial nuclear protein binding to -45/-19 PRD III eNOS 5'-flanking region and competitor oligonucleotides. Lane 1 represents -45/-19 probe alone. Lane 2 represents -45/-19 incubated with 10 μg of BAEC nuclear extract. In lane 3-5, 100X molar excess of various competitors were incubated with the binding reaction: -45/-19 (lane 3), -61/-27 (lane 4), and Flt-1-E4 (lane 5). 2.5 X10^4 dpm of labelled -45/-19 probe was used in the binding reaction. Arrows on left represent protein/DNA complexes.
Table VIII. Sequence alignment of the 3' → Ets site in PRD III of eNOS and E4 Ets site of Flt-1 promoters.

<table>
<thead>
<tr>
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<th>eNOS PRD III (-33/-24)</th>
<th>Flt-1 E4 (-57/-48)</th>
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the rat stromelysin 1 promoter site (Ets-2) (331) and the polyomavirus PEA3 site (PEA3) (330) and HIV-2 LTR (Elf-1) (179) also competed away all protein/DNA complexes (data not shown).

To explore the importance of the palindromic Ets sites (-37/-34 (5'→) and -30/-27 (3'→)) present in PRD III, mutated forms of the -45/-19 probe were designed (Table VII) and assessed with EMSA analysis. Mutant probes incorporating a 5'→ Ets site mutation or a 3'→ Ets site mutation in PRD III were used. Mutation of the 5'→ Ets site in PRD III evidenced a reduction in protein/DNA complex J formation (Figure 56. lane 3). In contrast, mutation of the 3'→ Ets site did not abrogate complex J formation, but rather enhanced it (Figure 56. lane 4). Therefore, these mutant probes demonstrated that complex J requires a functional 5'→ Ets site to form on PRD III and basal binding of complex J is inhibited by the 3'→ Ets site. Mutating the 5'→ Ets site reduced the formation of complex J (Figure 56. lanes 3), however mutating the 3'-end site did not (Figure 56. lane 4).

Mutant probes were also utilized as competitor oligonucleotides in EMSA assays (Figure 57). All oligonucleotides (wild type and mutants) were able to specifically and effectively inhibit complex H and I formation with the wild type probe (100 fold molar excess of competitor oligonucleotides was used each containing various mutations). Exposure to non-specific oligonucleotides showed no competition (data not shown). Mutation of both the 5'→ and 3'→ Ets sites in PRD III resulted in a loss of competition of complex J, when used in 100-fold molar excess (Figure 57. lane 4). Mutation of the 5'→ Ets site also minimized the ability to compete away complex J (Figure 57. lanes 5). Similarly, mutation of the 3'→ Ets site minimized the competition for complex J (Figure 57. lane 6). A mutant oligonucleotide was also designed such that the 5'→ Ets site was mutated so as to eliminate the palindromic Ets sites (5'→/3'→) in
Figure 56. EMSA of endothelial nuclear protein binding to wild-type and mutant -45/-19 PRD III eNOS probes. Lane 1 represents -45/-19 probe alone. Lane 2 represents -45/-19 incubated with 10 μg of BAEC nuclear extract. Lane 3 represents -45/-19 (mut 5' <-- Ets) incubated with 10 μg of BAEC nuclear extract and lane 4 represents -45/-19 (mut 3' --> Ets) incubated with 10 μg of BAEC nuclear extract. 2.5 X10^4 dpm of labeled probe was used in each binding reaction. Arrows on left represent protein/DNA complexes.
Figure 57. EMSA of endothelial nuclear protein binding to -45/-19 PRD III eNOS probe and exposure to mutated competitor oligonucleotides. Lane 1 represents -45/-19 probe alone. Lane 2 represents -45/-19 incubated with 10 μg of BAEC nuclear extract. In lane 3-7, 100X molar excess of various competitors were incubated with the binding reaction: -45/-19 (lane 3), -45/-19 (mut 5' <-- / 3' --> Ets) (lane 4), -45/-19 (mut 5' <-- Ets) (lane 5), -45/-19 (mut 3' --> Ets) (lane 6) and -45/-19 (mut-dir-Ets) (5' --> Ets / 3' --> Ets) (lane 7). 2.5 X10^4 dpm of labeled -45/-19 probe was used in the binding reaction. Arrows on left represent protein/DNA complexes.
PRD III and replacing them with a direct repeat or reiterated Ets sites (5' →/3' →). Exposure of this direct repeat mutant oligonucleotide as a competitor (Figure 57, lane 7) resulted in enhancement of complex J formation. Therefore, these mutant oligonucleotide competition results are consistent with the labeled mutant probe results, suggesting that complex J formation is dependent on the palindromic Ets sites in PRD III, especially the 5' ↔ Ets site. Moreover, binding is modulated by orientation of the Ets sites and inhibited by the 3' → Ets site. In summary, studies of endothelial cell nuclear extracts and double-stranded oligonucleotide probes spanning the PRD III functional domain demonstrate nucleoprotein complexes composed of Sp1, variants of Sp3, MAZ and Ets family members (perhaps Elf-1, PU.1 and Fli-1). A minority of some of the protein/DNA complexes seen at this EMSA resolution have not been accounted for and thus future experiments are needed to assess the protein composition of the remaining minor protein/DNA complexes.

V.4.4 TRANS-ACTING FACTOR INTERACTION STUDIES USING IN VITRO DNASE I FOOTPRINTING

In order to map the relative binding of endothelial and non-endothelial nuclear extracts to the eNOS promoter region, specifically PRD III. in vitro DNase I footprinting analysis was performed (Figure 58). A DNase I footprinted region was detected using HUVEC but not HeLa nuclear extract (n=3). Surprisingly, this footprint broadly extended from −54 to −3 relative to the start site of transcription. This footprinted region included the 20 bp that account for PRD III
Figure 58. In vitro DNase I footprinting analysis of the human eNOS promoter using HUVEC and HeLa nuclear extracts. Lane 1 represents the pGL2-1193/+109 construct incubated with HUVEC nuclear extract and subsequently subjected to DNase I digestion. Lane 2 represents pGL2-1193/+109 construct alone digested with DNase I and lane 3 represents the pGL2-1193/+109 construct incubated with HeLa nuclear extract. Digested DNA fragments were subjected to arithmetic linear PCR using a labelled antisense eNOS primer (see Methods). Representative results are depicted (n=3). Similar findings were evident using a sense primer (data not shown). Black bar illustrates footprinted region. PRD III is indicated.
as well as regions downstream of PRD III. Comparable findings were detected with the sense strand (data not shown, n=3). Future studies involving in vitro DNase I footprinting analysis using recombinant proteins must be used to identify the trans-factors interacting with this footprinted region. In addition, in vivo footprinting, though technically demanding, will be revealing. The absence of a footprint in the HeLa lane may give insight into the role of PRD III in preinitiation complex formation in vascular endothelial cells.

V.4.5 RUN-OFF TRANSCRIPTION ANALYSIS (107)

Chapter II outlined the considerable activity demonstrated by eNOS promoter/reporter constructs in a subset of non-endothelial cells (including HeLa and HepG2). In order to assess whether the human eNOS promoter is transcriptionally active in endothelial versus non-endothelial cells nuclear run-off analysis was performed. In nuclear run-off assays, transcriptionally engaged RNA pol II complexes in isolated nuclei continue to elongate RNA for an average of 100 to 200 nt in the presence of radioactively labeled ribonucleotides. Run-off transcripts were labeled from isolated nuclei of HUVEC, HeLa and HepG2 followed by hybridization to unlabeled eNOS DNA probes spanning different regions of the gene. The nuclear run-off data for human eNOS suggested that the native human eNOS gene was transcriptionally engaged in both endothelial and non-endothelial cells (Figure 59). HUVEC samples demonstrated a clear difference in RNA pol II loading at the 5’-end of the gene compared to the 3’-end. In addition, RNA pol II loading appeared to be especially dense over exon 1 in HUVEC as compared with HeLa and HepG2. Densitometric analysis indicated that the signals at the 5’-end in HeLa were approximately 20% that of HUVEC respectively (n=3). However, the signal generated using the 3’-cDNA probe, which represents elongation of transcripts already
Figure 59. Transcriptional activity of the human eNOS gene in endothelial and non-endothelial cells. Nuclei were isolated and $^{32}$P-labelled nuclear run-on products hybridized to linearized, denatured genomic and cDNA probes slotted onto nitrocellulose filters. Probes included the plasmid DNA pBluescript, and human GAPDH cDNA as negative and positive controls respectively. The human eNOS 5'-specific probe was a BamHI/Smal genomic fragment spanning from -530 through +780 with respect to the start site of transcription, the mid-region probe was a 1.1 kb EcoRI cDNA fragment spanning exons 8 through 16 and the 3'-probe was a 1.7 kb KpnI cDNA fragment spanning exons 17 through 26. Shown is a representative run-off from 4 independent experiments with consistently reproducible results.
approaching completion, served as a more accurate measure of the RNA pol II complexes transcribing full-length mRNA. Although these results argue that there are comparable levels of RNA pol II complexes loading the 3' end of the eNOS gene, generating full-length transcript in both endothelial and non-endothelial cells, there is a clear difference in the levels of RNA pol II complexes at the 5' end of the gene. These differences in levels of RNA pol II processivity may provide another mechanism of regulation for human eNOS gene expression.

V.4.6 TRANSIENT TRANSFECTION FUNCTIONAL STUDIES IN DROSOPHILA SCHNEIDER SL2 CELLS

V.4.6.1 Sp1 and Ets family members are essential activating components of PRD I, II and III

Functional promoter analyses in endothelial cells revealed that mutating activator regions encompassing the low-affinity Sp1/Sp3 recognition sequence (-45/-40) resulted in a reduction of eNOS promoter/reporter activity (Figure 48). Similarly, mutating the region encompassing a palindromic Ets site repeat (-61/-27 and -45/-19) also resulted in a reduction of promoter activity (Figure 48). Based upon this background, the model proposed in Chapters II and III, wherein Sp1, Sp3 and Ets family members are essential for in vivo eNOS promoter function can be extended to include PRD III. To evaluate this hypothesis, a series of transient transfection experiments were performed in cells which lack constitutive Sp1, Sp3 and Ets activities, namely the Drosophila Schneider cell line (n = 3, triplicate determinations) (63, 117).
Though threshold amounts of Ets-1, Sp3 and Sp1 expression cassettes (5 ng) alone had minimal effects on the pGL2-1193/+109 luciferase reporter construct (Figure 21. Chapter III) (n=3, triplicate determinations), the combined addition of threshold amounts of Ets-1, Sp3 and Sp1 activated the eNOS promoter in a cooperative fashion (Figure 21. Figure 60). These results highlight the cooperative and complex nature of Ets-1, Sp3 and Sp1 interactions in functional eNOS promoter activity and underscore their essential contributions to nucleoprotein complex formation for the eNOS promoter. To demonstrate that these factors were acting through cis-DNA sequences found in PRD I, II and III, these factors were co-transfected with 6 linker-scanning mutant constructs. Co-transfection of the PRD I linker-scanning mutant construct with threshold amounts of Sp1, Sp3 and Ets-1 demonstrated an approximate 80% decrease in activity relative to the activity of the wild type eNOS construct (Figure 60). When activator sequences between -144 and -135 in PRD II were mutated an approximate 75% decrease in functional promoter activity was observed confirming the important contribution of 5'-regions of PRD II.

When sequences corresponding to the Ets recognition site in PRD II were mutated, an approximate 50% decrease in functional promoter activity was observed (Figure 60). When sequences between -124 and -115 were mutated, this resulted in a 45% decrease in functional promoter activity (Figure 60). Mutating sequences accounting for PRD III had similar results. Mutating the low-affinity Sp1 site and the 5'-Ets site in PRD III, -44/-35mut, resulted in a 32% decrease in functional promoter activity. However, mutating the sequences from -34/-25 did not affect eNOS promoter activity in SL2 cells (Figure 60). Taken together, these results identify PRD I, II and PRD III as critical activator recognition sequences for eNOS promoter function. Mutating the Sp1/Sp3 sites in PRD I, II and III, and mutating the Ets site in PRD II and the 5'-Ets site in PRD III resulted in dramatic decreases in functional promoter activity both in endothelial cells and the Drosophila Schneider heterologous expression system.

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Figure 60. Sp1, Sp3 and Ets-1 transactivate human eNOS promoter/reporter luciferase constructs in Drosophila Schneider cells. Assay of promoter activity of wild-type and linker-scanning mutant pGL2-1193/+109 promoter/reporter luciferase constructs following co-transfection with threshold amounts of pPacUSp1 (5 ng), pPacUSp3 (5 ng) and/or pPacUEts-1 (5 ng). Shown are average of experiments (triplicate determinations), performed 4 times. Data are expressed as fold increase in luciferase activity ± S.E.M. relative to pGL2-1193/+109.
Given the demonstration that MAZ accounts, in part, for endothelial PRD III nucleoprotein complexes we determined the functional properties of MAZ in endothelial co-transfection experiments (Figure 61). CMV-directed expression of MAZ protein in BAEC had a stimulatory effect on the -1193/+109 eNOS promoter/reporter construct in transient transfection assays. This augmentation of eNOS promoter activity occurred over a range of added MAZ heterologous eukaryotic expression cassette (100 ng to 1 µg). MAZ enhanced eNOS promoter activity 3.7 to 37.6 fold above expression vector alone (n = 5, triplicate determinations, 100 ng).
Figure 61. MAZ transactivates the human eNOS promoter in BAEC. MAZ augments pGL2-1193/+109 promoter/luciferase construct activity in BAEC (pCGN-MAZ, 100 ng). Data are expressed as fold increase in luciferase activity ± S.E.M. relative to pGL2-1193/+109. Shown are results from 5 independent experiments (triplicate determinations) express as fold increase in luciferase activity ± S.E.M. relative to empty expression vector alone.
Two tightly clustered activator regions were identified in the proximal promoter regions of the human eNOS gene using deletion analysis and linker-scanning mutagenesis: positive regulatory domain I (PRD I) (-104/-95) and PRD II (-144/-115) (see Chapter II) (156). Generation of a further series of linker-scanning mutants downstream of -45 identified a third positive regulatory domain, termed PRD III. Positive regulatory domain III spans a 20 bp region from -44 to -25, relative to the start site of transcription. The high degree of relatedness of PRD I, II and III in the human, bovine and murine promoter sequences reinforced the significance of these domains in the transcriptional regulation of the eNOS gene (Figure 62). PRD III was not detected with the deletion series (Chapter II) given that PRD I seems necessary but not sufficient for basal promoter activity. Thus, linker-scanning mutagenesis provides a method for refining functionally important cis-regulatory domains. Linker-scanning mutagenesis also provided evidence for the existence of a potential fourth regulatory domain, spanning sequences from +7 to +16, important in the regulation of eNOS promoter activity (Figure 48). This domain encompasses a negative regulatory region that is active in vascular endothelial cells. This mutagenesis approach altered the 5'-structure of the reporter RNA. To confirm that this increase in luciferase activity is a function of the amount of transcript produced from the construct, RNase protection assays or primer extension analysis may be performed to quantitate the levels of reporter transcript produced. If no difference in mRNA levels is detected between the wild type and mutant construct, then the increase in luciferase activity may be attributed to an enhancement in translation efficiency, rather than a direct effect on transcription initiation.
Figure 62. Cross-species alignment of the murine, bovine and human eNOS proximal promoter regions showing presence of PRD III. Enclosed sequences denote regions of nucleotide identity. Conserved transfactor binding sites and PRD I and II are illustrated. Human eNOS +1 site is illustrated.
Analysis of trans-factor binding and functional expression studies revealed a similar degree of cooperativity and complexity in PRD III function, as identified with PRD I and II in Chapters II and III. Electrophoretic mobility shift assays performed with probes spanning PRD III demonstrated specific protein/DNA complex formation. Specific evidence for the important and complex contributions of Sp1 family members to eNOS promoter activity was evident. EMSA and functional studies suggested the presence of a low-affinity Sp1 site [5'-CCTCCC-3'] at positions -45 to -40 in PRD III. An oligonucleotide spanning this region of PRD III (-61/-27) was also recognized by Sp3 variants. Various Ets family members were shown to interact with PRD III using EMSA analysis. Specifically, proteins that are antigenically-related to Elf-1, PU.1 and Fli-1 are present in specific protein/DNA complexes formed on PRD III. A shift abrogation was evident with the addition of anti-Elf-1, and supershifted complexes were evident upon the addition of anti-PU.1 and anti-Fli-1. The different effects of antibodies may be due to varied affinity of each antibody for their respective protein and/or may reflect the abundance of the each Ets factor (Elf-1 vs. PU.1 vs. Fli-1) in endothelial cell nuclear extracts. In contrast, Chapter III demonstrated no effect on nucleoprotein complex formation with PRD II probes upon the addition of anti-PU.1 and anti-Fli-1. This may be indicative of varied Ets family members interacting with different regulatory regions of the eNOS promoter. Although conclusive evidence demonstrating Ets-1 interaction with PRD III was not shown, functional studies in Drosophila demonstrated the functional contribution of Ets-1 to PRD III function. The acknowledged difficulties with Ets family member antisera and cross-competition among Ets binding sites is known. Moreover, an autoinhibitory domain of Ets-1 can inhibit important protein-protein or protein-DNA interactions in EMSA studies. This has led to the realization that the Ets-1 protein is negatively regulated through conformational changes involving intramolecular interactions (151). Specifically, an inhibitory module allosterically modulates the
DNA-binding activity of Ets-1. Activation of Ets-1 requires a conformational change and this autoinhibition of Ets-1 can be relieved by either protein partner(s) or post-translational modifications (151). This is supported by work performed by Seth et al (283) where addition of a monoclonal antibody directed against the ‘repression domain’ of Ets-1, induced a conformational change resulting in the increased accessibility of the DNA-binding domain of Ets-1 and increasing its affinity to the GATA-1 promoter.

MAZ was also shown to interact with PRD III. Addition of an antibody directed against MAZ resulted in the shift abrogation of many of the slower migrating complexes formed with the \(-61+/+27\) PRD III probe (Figure 52, lane 4). MAZ was also shown to functionally interact with PRD II (Chapter III). MAZ has received increasing attention for its protean roles in gene regulation: transcription initiation, interference and termination (33). MAZ is especially important in TATA-less promoters (243). Particularly well-studied examples of MAZ involvement in transcriptional initiation include \(c\)-myc, the adenovirus major late promoter, the serotonin 1a receptor and CD4 (33, 80, 243). Transient transfection analysis in bovine aortic endothelial cells demonstrated that MAZ is capable of activating the eNOS promoter in endothelial cells (Figure 61). This supports a role for MAZ in regulating preinitiation complex formation for the eNOS gene in endothelial cells. However, MAZ was shown to exert a repressive effect on Sp1-mediated and Sp1/Sp3/Ets-1-mediated promoter activation in \textit{Drosophila} Schneider cells (Chapter III). Further studies will be necessary to understand why the contribution of MAZ to transcriptional regulation of the eNOS gene differs between \textit{Drosophila} Schneider cells and endothelial cells. It may be possible that MAZ requires a co-activator in order to demonstrate activation potential. A further possibility may be a requirement for post-translational modification of MAZ for it to function as an activator. It is plausible that
such a pathway(s) may not be functional in *Drosophila* and MAZ protein may be in a conformation that sterically inhibits its capabilities of functioning as an activator.

PU.1 is an Ets family member expressed in a B-cell and macrophage-specific manner (167). The PU.1 protein contains three domains in its protein structure, including the C-terminal ETS domain, the N-terminal activation domain and a domain containing PEST sequences (145). PEST sequences (proline-glutamic acid-serine-threonine) play a role in ubiquitin-mediated protein degradation (261). The PEST sequences are also involved in protein-protein interactions with the B-cell specific nuclear factor, NF-EM5 or PIP (251). Phosphorylation of these sequences has been shown to preclude this protein-protein interaction *in vitro* (252). PU.1 has not been shown to be expressed in endothelial cells (132). PU.1 has been shown to be a regulator of myeloid-cell-specific genes, such as CD11b (238), M-CSF receptor (357) and c-fes (124). In all three promoters, PU.1 interacts with binding sites that are located just 5' of the start site of transcription. PU.1 has also been shown to physically interact with TBP *in vitro* (116). Based on these findings, one may propose a model wherein Ets family members facilitate the recruitment of essential components of the basal transcription machinery such as TFIID to efficiently initiate transcription. As described in the introductory section of this thesis, the human eNOS promoter does not contain a consensus TATA box. Thus, Ets factors may be implicated in the recruitment of TFIID to the eNOS core promoter to begin the process of the preinitiation complex formation.

Fli-1 protein is a member of the Ets family that is preferentially expressed in hematopoietic cells (166). Fli-1 expression has also been detected in endothelial cells (198, 210). Retroviral insertionional activation of the Fli-1 gene has been observed during Friend murine leukemia virus-
induced erythroleukemia. Insertional activation of Fli-1 is the first detectable genetic alteration in this erythroleukemia (20, 131). PU.1 is also dysregulated by Friend leukemia virus insertion. Fli-1 is also activated in Ewing's sarcoma upon chromosomal translocation to chromosome 22 (11q24:22q12) (71). This creates a novel fusion protein in which the DNA binding domain of Fli-1 is fused to the RNA binding protein EWS. Fli-1 is likely to play an important role in vascularization and hematopoiesis since overexpression of Fli-1 in Xenopus embryogenesis results in the frequent absence of circulating red blood cells and ectopic differentiation of mature erythrocytes in vesicles not connected to the vascular system (73, 265). Fli-1 has also been shown to promote angiogenesis (73, 265). Two forms of Fli-1 exist. Fli-1 and Fli-1b. Fli-1b is generated by differential splicing events and alternative promoter usage (75). Fli-1b has transcriptional activation properties similar to those of Fli-1 (75). Fli-1 has been shown to regulate a number of genes including human heme oxygenase-1 (73), murine Egr1 (332) and the megakaryocyte glycoprotein IX gene (173).

Ets family members are classical activators that stimulate transcription from a distance but they may also have a primary role in the formation of the initiation complex on minimal core promoters lacking a TATA sequence. such as eNOS. Thus, the palindromic Ets sites in PRD III may bind Ets factors that function as important components of the general transcription machinery. The concept of Ets factors playing a role in transcription initiation is not a novel one. For example, the cytochrome c oxidase subunit IV nuclear gene, which is TATA-less. has two Ets binding sites close to the first major initiation site. These elements bind Ets factors and are required for promoter activity (322). Another example involves the thymidylate synthetase gene which contains an Ets motif [5'- CCG GAA G -3'] close to the initiation sites and is essential for activity (149).
Palindromic Ets sites have been identified in other promoters, such as the GATA-1 promoter (283), human stromelysin promoter (165) and the p53 promoter (312) (Figure 63). The Ets sites in the rat stromelysin promoter have been shown to interact with Ets-1 protein, however, the sites were reported to bind non-cooperatively (331). Studies performed on the GATA-1 promoter revealed that both Ets binding sites in the palindrome were required for efficient DNA-binding (283). Furthermore, for efficient binding, the two Ets sites must be palindromic, as opposed to a direct repeat. Ets binding sites in the same orientation resulted in a decreased binding to the DNA (283). Studies performed with the p53 promoter assessed the effect of differential spacing between the palindromic Ets sites (319). This group observed an inverse correlation between an increasing distance in between the Ets palindrome and Ets-1 and Ets-2 DNA binding activity. Ets-1 and Ets-2 bind to the palindromic Ets sites with greater efficiency when the binding sites are separated by 4 nucleotides (natural promoter setting) as opposed to an increased distance (up to inserted 20 nucleotides) (319). In contrast, Fli-1 demonstrated increased binding affinity at a 20 nucleotide spacing and no binding at the 4 nucleotide spacing. This provides another level of specificity for targeting varied Ets members to a promoter (127, 319), perhaps relevant to the eNOS story.

Given this background it is of great interest to examine the functional importance of each of the two Ets sites in PRD III with respect to the relative contribution of each to functional promoter activity in endothelial cells and a heterologous expression system (Drosophila), the relative orientation of each site (palindromic versus reiterated) and the spacing between each site. Results of the current work demonstrate intriguing changes in EMSA protein/DNA complexes that form upon PRD III depending upon whether the 5’ ← or 3’ → Ets sites are mutated. It is of interest
Figure 63. Comparison of palindromic/dual Ets sites in various genes.

<table>
<thead>
<tr>
<th>GENE</th>
<th>PALINDROMIC ETS SITES (nucleotide position relative to +1)</th>
<th>SPACING BETWEEN ETS SITES (N)</th>
<th>ORIENTATION OF PURINE BOX (GGAA) =</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human eNOS</td>
<td><strong>TCTTCTAA------GGAAAA</strong> (-39/26)</td>
<td>3</td>
<td>← →</td>
<td>[Marsden et al, J. Biol. Chem. 268:17478,1993]</td>
</tr>
<tr>
<td>Human platelet glycoprotein Ibα</td>
<td><strong>AAGGAAAGC------GGAGGA</strong> (-151/137)</td>
<td>3</td>
<td>→ →</td>
<td>[Hashimoto et al, J. Biol. Chem. 270: 24532, 1995]</td>
</tr>
<tr>
<td>Human stromelysin</td>
<td><strong>CAGGAAGCAC------TTCCCTG</strong> (-202/201)</td>
<td>4</td>
<td>→ ←</td>
<td>[Kirsteinet al, J. Biol. Chem. 271: 18231, 1996]</td>
</tr>
<tr>
<td>Human vWF</td>
<td><strong>ATTTCCTTTCTATTGT------TTCCCT</strong> (-57/37)</td>
<td>9</td>
<td>← ←</td>
<td>[Schwachtgen et al, Oncogene 15: 3091, 1997]</td>
</tr>
<tr>
<td>Human mitochondrial ATP synthase β-subunit</td>
<td><strong>CAGGAATCGGCCCTC------TTCCCTA</strong> (-303/282)</td>
<td>10</td>
<td>← ←</td>
<td>[Villena et al, J. Biol. Chem. 269: 32649, 1994]</td>
</tr>
<tr>
<td>Chicken GATA-1</td>
<td><strong>GAGGAGGGCAGATGCCCTCT</strong> (-503/480)</td>
<td>12</td>
<td>← ←</td>
<td>[Seth et al, Oncogene 8: 1783, 1993]</td>
</tr>
</tbody>
</table>
that mutating the $3' \rightarrow$ Ets sites has a major effect on promoter function in BAEC but no effect on the ability of Sp1/Sp3/Ets-1 to transactivate the promoter in Drosophila SL2 cells.

Through analysis of nucleoprotein complexes in endothelial cells and functional domain studies in Drosophila Schneider cells, we demonstrated positive protein-protein cooperativity involving Sp1, variants of Sp3 and various Ets family members (Chapter III). To elucidate whether this cooperativity is also dependent on a functional PRD III domain, heterologous Drosophila expression studies were performed with the linker-scanning mutants that account for PRD III, namely $-44/-35\text{mut}$ and $-34/-25\text{mut}$. As predicted, mutating the $5'$- end of PRD III (which is comprised of the low affinity Sp1 site and the $5'$- Ets site) resulted in a functional decrease in promoter activity demonstrated by threshold amounts of Sp1, Sp3 and Ets-1. Interestingly, mutating the $3'$-end of PRD III did not have an effect on the positive interaction between Sp1, Sp3 and Ets-1 in Drosophila. One explanation for this finding may be that Ets-1-mediated transactivation is not mediated through the $3'$- Ets site in PRD III. Cotransfection experiments using Elf-1, PU.1 or Fli-1 expression constructs need to be performed to assess the importance of these factors in function of PRD III.

Nucleoprotein analysis, using the $-45/-19$ probe revealed informative findings. Complexes H and I likely represent complexes containing Ets factors since various Ets oligonucleotide competitors were very effective in competition studies. Mutating either one of the two palindromic Ets sites in PRD III alone did not effect the formation of complexes H and I. A more complex issue relates to why competition is observed with the unlabeled $-45/-19$ (mut $5' \bullet / \text{mut } 3' \rightarrow$) oligonucleotide. Lack of competition was evident with non-specific oligonucleotides. We posit that H and I represent multiprotein complexes that consist of Ets family members, but also other
complexes. The binding of the nucleoprotein complexes to the -45/-19 probe must therefore also reflect interaction with cis-DNA elements on the probe other than the core Ets motif. We posit that members of the general transcriptional machinery may also be interacting with PRD III through complexes H and I. For example, TFIIID may be interacting with PRD III and playing a role in preinitiation complex formation. This is supported by the reported interaction between PU.1 and a member of the TFIIID complex, namely TBP (116). Future studies using recombinant proteins and antibodies against various general transcription factors, such as TBP, will be required to examine this model further.

Complex J formation is dependent on a functional 5' → Ets site since mutating this site abrogated formation of complex J. Using the mutated 3' → site oligonucleotide as a probe enhances formation of complex J. This implies that basal binding of complex J is inhibited by the 3' → Ets site and may reflect that unique members of the Ets family are recruited to the unique topology of eNOS PRD III activator regions. This concept is also supported by competition studies performed with the direct repeat mutated oligonucleotide (5' → Ets/ 3' → Ets). Exposure of this cold competitor to the binding reaction resulted in titration of the factors binding to the 3' → Ets site. By removing the factors that bind to the 3' → Ets site, proteins involved in complex J formation are capable of interacting with the wild type probe and hence an increase in complex J formation is observed. This is consistent with the work published in the GATA-1 promoter where decreased binding affinity for Ets-1 was observed with a direct repeat as opposed to palindromic Ets sites (283). These EMSA results correlate with the lack of effect of the mutation on the ability of Sp1/Sp3/Ets-1 to transactivate the promoter in Drosophila SL2 cells where the 3' → site is mutated (Figure 60). Mutating the 3' → site enhanced the ability for trans-factors to bind to the 5' ↔ Ets site and activate the eNOS promoter. Future studies are
necessary to further address the effect of differential spacing of the palindromic Ets sites in PRD III.

This correlates with the model for eNOS promoter activity, which describes the cooperative binding and interaction between PRD I, II and III trans-factors for efficient enhanceosome formation and preinitiation complex formation for subsequent eNOS transactivation. Future studies need to address this issue further by using longer oligonucleotides spanning PRD I, II and III in EMSA analysis and assessing the dependence of intact binding sites for the various Ets factors.

DNase I footprinting analysis revealed a footprint spanning PRD III on both the top and bottom strands. Interestingly, the footprint extended past PRD III towards the start site of transcription. This suggests that proteins interacting with PRD III are involved in preinitiation complex formation such that the preinitiation complex (PIC) that forms over the PRD III region inhibits DNase I accessibility to the DNA. The PIC does not interact directly with the promoter region downstream of PRD III since mutating these regions did not have a functional effect on promoter activity.

The nuclear run-off results are of great interest in view of the footprinting results. Nuclear run-off analysis revealed a surprising difference in RNA pol II loading at the 5'-end of the eNOS gene compared to the 3'-end in HUVEC. This may be indicative of a unique mechanism of regulating eNOS gene expression in endothelial cells. This 5'- polarity in RNA pol II density is often generated by the resumption of elongation during the nuclear run-off procedure of RNA polymerase complexes that are paused in upstream regions. This RNA pol II loading mechanism
is only observed in endothelial cells, since analysis using HeLa and HepG2 extracts did not reveal similar results (Figure 59).

This selective 5’- loading of RNA pol II in HUVEC should not be confused with attenuation, which is the process by which transcriptional pausing and termination is used to modulate the expression of a gene. For example, c-myc (25), c-myb (21, 22), HIV-1 (154) and CSF-1 (43) gene expression is controlled at the level of transcriptional attenuation. Transcriptional attenuation is detected with nuclear run-on assays. These assays measure the distribution of transcription complexes within a gene at the time of nuclear isolation. Essentially, transcriptionally engaged RNA polymerase II complexes in isolated nuclei continue to elongate nascent RNA in the presence of radioactively labeled ribonucleotides. The labeled mRNA is then hybridized to probes that span different regions of the gene of interest. Such studies reveal selective 5’- RNA pol II loading compared to 3’- RNA pol II signals. The mechanisms underlying promoter proximal pausing of RNA polymerase II transcription complexes are unknown. However, many models have been proposed. Secondary structure of nascent mRNA may play a role in transcriptional attenuation. For example, polythymidine/polyuridine sequences which are preceded by a GC-rich sequence of dyad symmetry encode RNA with the potential to form a stem-loop structure (298). These are structural features present in simple transcription termination signals and are commonly used in prokaryotic systems (298). Such sequences are present in the first intron of the c-myb gene (307), the TAR region of the HIV-1 gene (66), as well as the human c-myc gene (26). Specific sequences may also play a role in pausing of the RNA polymerase II transcription complex. These sequences may be DNA or RNA cis-elements. For example, the HIV-1 gene contains a bipartite DNA element named IST (inducer of short transcripts) (259). This element is located downstream of the HIV-1 start site of transcription.
and is thought to direct assembly of transcription complexes that are incapable of efficient elongation (288). Specific trans-factors may also physically block the transcription elongation complex. MAZ protein has been shown to bind to the ME1a1 cis-element located in the first intron of the c-myc gene. This element has been shown to regulate transcriptional termination (33). Perhaps, binding of MAZ protein to this cis-element creates a physical block that prevents the transcriptional elongation complex from proceeding to downstream sequences. Chromatin structure may also influence transcriptional pausing or processivity (170). The linker histone H1 or other factors may be involved in the formation of repression loops, which will trap or arrest transcription complexes (170). In murine B lymphoid tumors, a major DNase I-hypersensitivity site (site IV) has been mapped near the transcription block of the c-myb gene (21, 22). This hypersensitivity site is more readily detected when attenuation efficiency is increased. This suggests that changes in higher order chromatin structure may accompany changes in attenuation (310). The phosphorylated state of the carboxyterminal domain (CTD) of RNA polymerase II may also contribute to the promoter-proximal pausing of the transcription complexes. CTD hyperphosphorylation normally accompanies the transition from initiation to elongation (248). O'Brien et al demonstrated that for genes containing a 5' paused polymerase, passage of the paused RNA polymerase into an elongationally competent mode in vivo coincides with phosphorylation of the CTD (231). Therefore, modifications to RNA polymerase II or other components of the transcription complex would result in the conversion of a less processive to a more processive elongating transcription complex. For example, TFIIS associates with the transcription complex after initiation. This factor provides the transcription complex the ability to overcome intrinsic sites of pausing and premature termination, as well as to read through blocks to elongation caused by bound protein complexes (263). Thus, absence of such a factor may contribute to the attenuation of the elongating transcription complex.
The regulation of transcriptional elongation is now recognized as an important component in the control of eukaryotic gene expression. Factors that regulate transcription elongation complexes and their ability to attenuate transcription still need to be clarified. Regulating the processivity of the RNA polymerase II-transcription complex is the rate-limiting step that is required for ensuring that a gene is only expressed when appropriate factors that control transcription elongation are present.

Taken together, a model may be proposed. Activators known to enhance the rate of transcription (Sp1, variants of Sp3, Ets family members, MAZ, YY1) may act not only at the level of transcription initiation but also at the level of transcription elongation. Nonprocessive transcription complex formation is a mechanism in which genes poise themselves ready to be expressed. In other words, a transcription complex may be already assembled on the promoter, but it lacks the essential components or modifications to carry out promoter clearance. Through the action of activators and enhancers, these nonprocessive transcription complexes are converted to a more processive form that is now capable of performing efficient elongation. The mechanism of the transition process from a nonprocessive form to a processive form is still unknown. However, contributions from the presence/absence of certain elongation factors, the phosphorylation state of the CTD of RNA polymerase II, as well as chromatin structure have been suggested.

In summary, we posit that positive regulatory domain III is involved in preinitiation complex formation. Functional Ets sites are required for Ets protein interaction with PRD III. We posit that Ets factors aid in the recruitment of members of the general transcriptional machinery, such
as TFIID, and facilitate efficient preinitiation complex formation in vascular endothelial cells. Formation of the enhanceosome on upstream promoter regions (such as PRD I and II) and the subsequent interaction with the preinitiation complex formed on PRD III serves to elicit phosphorylation of the CTD of RNA pol II and subsequent promoter clearance of the holoenzyme complex. Sp1, Sp3, MAZ and Ets family members participate in this interaction in order to assemble the appropriate machinery for efficient transcription initiation. We posit that PRD III plays an important role in TFIID binding and preinitiation complex formation.

However, a paradox must be addressed. In Chapter IV, analysis of the methylation status of the eNOS gene in HepG2 and HeLa cells revealed extensive methylation across the proximal promoter, but nuclear run-off analysis presented in this chapter revealed evidence for eNOS mRNA transcript formation. Although, the eNOS promoter in HepG2 and HeLa was densely methylated, hypomethylation footprints were evident (207), specifically spanning PRD I (Figure 37). Perhaps the reduced levels of methylation of DNA spanning PRD I reflect, in part, Sp1 family members binding and partial activation of the preinitiation complex thus permitting a low level of eNOS pre-mRNA transcript formation in these non-endothelial cell types. However, only the combined contribution of a functional PRD I and II (contributing to formation of the enhanceosome) and PRD III (contributing to formation of the preinitiation complex) will result in the robust expression of eNOS evidenced by HUVEC nuclear extracts. This is supported by the complete lack of methylation of the eNOS promoter in varied endothelial cells. Experiments utilizing episomes may not address the full story and thus chromatin-based studies need to be performed.
Future experiments are needed to explore the role of PRD III in the preinitiation complex formation. Specifically understanding the function of varied Ets factors in the regulation of the human eNOS gene. Understanding the novel mechanism of regulating eNOS expression in endothelial cells will ultimately be critical for the development of potential therapies for diseases, perhaps through the development of novel gene transfer therapies.
CHAPTER VI

SUMMARY AND FUTURE STUDIES
CHAPTER VI. SUMMARY AND FUTURE STUDIES

VI.1 SUMMARY

In summary, this thesis demonstrated an unexpected complexity in the regulation of eNOS gene expression. We propose a model in which Sp1, variants of Sp3, and various Ets family members are subunits of a highly ordered ‘enhanceosome’ structure which is responsible for the activation of eNOS transcription in endothelial cells. Synergy between these trans-acting factors is likely to require multiple protein-protein and protein-DNA interactions. These trans-acting factors functionally cooperate within the enhanceosome to present to the basal transcriptional machinery a biochemical interface that is highly efficient with respect to transcription initiation. In the current work, coexpression of Sp1, Sp3 and Ets-1 in Drosophila Schneider cells enhanced transcription of eNOS promoter/reporter constructs compared to each factor alone. Mutating activator recognition sites for these factors, or removal of any of these factors, abolishes this synergy. This suggests that the enhanceosome structure is dependent upon protein-protein interactions between these factors as well as interactions between the trans-factors and their corresponding cis-elements. Following an initial binding of Sp1 to a high-affinity element in PRD I, other Sp1 molecules and variant Sp3 proteins are recruited through binding or tethering and interact with low-affinity elements in PRD II. The initial recruitment of Sp1 may also facilitate the binding of the Ets proteins to PRD II elements.

Expression studies performed in endothelial and non-endothelial cell types revealed that eNOS promoter/reporter constructs lacked cell-specificity, suggesting that there exist cell-specific
mechanisms that repress native eNOS gene expression in non-expressing cell types. We posited that DNA methylation played a role in this cell-specific expression. Sodium bisulfite genomic sequencing revealed a different methylation pattern of the eNOS promoter in endothelial versus non-endothelial cell types. Transient transfection of in vitro methylated promoter/reporter constructs performed revealed an important role for methylation in transcriptional repression. DNA hypomethylation studies performed with 5-azacytidine resulted in increased expression of eNOS mRNA in cell types that do not normally express the eNOS mRNA transcript. The role of epigenetic regulation in cell-specific eNOS gene expression is a novel finding in the vascular endothelium.

Positive regulatory domain III is involved in preinitiation complex formation. Functional Ets sites are required for Ets protein interaction with PRD III. We posit that Ets factors aid in the recruitment of members of the general transcriptional machinery, such as TFIID, and facilitate efficient preinitiation complex formation in vascular endothelial cells. Enhanceosome formation on PRD I and II allows functional interactions with a preinitiation complex on PRD III and facilitates robust promoter clearance of the RNA pol II holoenzyme complex (Figure 64). Sp1, Sp3, MAZ and Ets family members are variably involved in the assembly of the appropriate biochemical machinery for efficient transcription initiation of the human eNOS gene.
Figure 64. Model of the transcriptional regulation of the human eNOS gene.
VI.2 FUTURE STUDIES

Not all protein/DNA complexes interacting with PRD III have been accounted for. Therefore, further experiments are needed to identify the remaining unidentified interacting proteins. This may be accomplished using recombinant proteins, competitor oligonucleotides and antibody addition. For example, to determine whether complexes H and I contain any general transcription initiation factors, recombinant proteins and antibodies may be used to demonstrate specific interaction with PRD III.

Future detailed biochemical analysis of protein-DNA and protein-protein interactions on the eNOS promoter will be needed to substantiate the enhanceosome model further. It will also be necessary to determine what alterations occur in eNOS enhanceosome structure and function in conditions known to be associated with biologically important alterations in eNOS mRNA expression.

In particular, enhanceosome formation is not only dependent on complex protein-protein and protein-DNA interactions, but also on highly stereospecific complex formation. Therefore, changes in helical phasing between PRD I, II and III should be detrimental for enhanceosome function. In order to assess this, the relative positions of these elements on the DNA double helix will be changed by inserting half- or full-length turns of DNA, using randomized DNA sequence, between the individual PRDs. A similar study was performed with the IFNβ gene enhancer (305). Transient transfections will then be performed in BAEC and assessed for promoter activity. We expect insertions of a half-helical turn will reduce promoter activity, whereas insertion of 10-12 bp, which reestablishes the relative positions of binding sites on the
face of the DNA helix, will fully restore the activity of the promoter. These experiments will suggest that the activators specifically contact the basal transcription complex or that the activators specifically contact each other.

To address whether the transcriptional synergy among the individual elements in PRD I, II and III are due to cooperative binding of the corresponding transcription factors, in vitro DNA binding experiments may be carried out using recombinant proteins. Various Ets family proteins will be in vitro transcribed and translated using the rabbit reticulocyte lysate system. These recombinant protein-containing extracts will then be used in EMSA analysis to demonstrate binding of specific Ets factors to PRD II and PRD III probes. Furthermore, these recombinant protein extracts, in combination with recombinant Sp1 family members may be used in quantitative DNase I footprinting analysis. Here, various combinations of recombinant proteins will be used to demonstrate cooperative binding and interaction with the three positive regulatory domains (PRD I, II and III). In addition, cooperativity could also be tested in the presence of an inserted half-helical turn between the PRD domains. Methylation interference assay could also be performed to map the protein/DNA contacts between the trans-acting factors and the PRD domains. To map the protein/DNA interactions in the context of a chromatin template, in vivo DNase I footprinting may be performed.

Finally, to determine if the helical constraints affect the transcriptional synergy demonstrated in Drosophila Schneider cells, these mutated constructs could be transiently transfected into SL2 cells with threshold amounts of Sp1, Sp3 and Ets-1. We predict that the synergistic and cooperative interactions between these factors will be abrogated in the presence of an altered DNA conformation in the eNOS promoter.
Future studies may also involve analyzing the function of the palindromic Ets sites in PRD III. For example, various oligonucleotides containing differential spacing between the Ets sites may be designed. In turn, the effect of increasing the distance between the Ets sites may be assessed using EMSA analysis utilizing both nuclear extracts and recombinant Ets proteins. We predict that altering the spacing of the Ets sites will decrease the affinity for Ets factors to bind to PRD III, consistent with the model of the enhanceosome.

After demonstrating the cooperative binding and assembly of the enhanceosome, the cooperative binding between the enhanceosome and the basal transcriptional machinery should be assessed. For example, in the Epstein-Barr virus promoter, BHLF-1, cooperative interaction between the enhanceosome complex (containing ZEBRA, HMG-1. -2 and cellular Sp1) and TFIIA/D and the holoenzyme have been demonstrated (82). Reciprocal cooperative interactions were shown among the activators and the general machinery in BHLF-1 gene regulation. Similarly, studies may be performed using the enhanceosome of eNOS and demonstrating cooperativity in preinitiation complex formation. Gel shift and DNase I footprinting studies will be used to elucidate the mechanism of interaction using purified TFIID. TFIIA and RNA pol II holoenzyme extracts. Also, in vitro transcription and protein recruitment assays with immobilized DNA templates and immunoblotting techniques to identify interacting proteins will be used to analyze the mechanisms of enhanceosome-dependent transcriptional synergy.

To assess the effect of PRD III mutations on preinitiation complex formation, stable cell lines of the various PRD III linker-scanning mutant constructs may be established. In turn, nuclear run-off analysis using nuclear extracts isolated from these stable cell lines will be performed. We
posit that marked differences in RNA pol II loading will be detected in the mutant vs. the wild type stable cell lines, demonstrating an important role for PRD III in preinitiation complex formation.

An intriguing aspect of eNOS gene expression is its relative restriction to the vascular endothelium. Possible transcriptional regulatory mechanisms involved were examined through a series of transient transfection assays with eNOS promoter/reporter constructs in a variety of endothelial and non-endothelial cells. The pGL2-1193/+109 promoter/reporter construct was found to be maximally active in both HUVEC and BAEC compared to the other non-endothelial cell types (Tables IIa, IIb and IIc). Interestingly, this construct was also found to be quite active in a number of non-endothelial cell types in which no appreciable eNOS mRNA transcripts were detected by RNase protection assays and northern blot analysis. Therefore, future experiments may be performed analyzing the deletion profile and linker-scanning mutant profile demonstrated by the non-endothelial cell types that exhibited unusually high promoter activity. This will give insight into the transcriptional regulation of the eNOS gene.

Chapter IV discovered a novel mechanism of regulation of the eNOS gene involving epigenetic contributions, specifically DNA methylation. In order to assess whether the eNOS promoter region is sufficient and required for DNA methylation-mediated repression, stable transfections of in vitro methylated promoter/reporter constructs should also be performed. Both endothelial and non-endothelial cell types will be stably transfected. Promoter activity will be assayed by luciferase expression and normalized to genomic copy number. These experiments will demonstrate whether a demethylation control region (DCR) is present on the regions of DNA transfected. For example, if the DCR is present on the construct, then the transfected DNA will
remain methylated in non-expressing cells and no promoter activity will be detected. On the other hand, the transfected DNA will become hypomethylated in genomic DNA isolated from endothelial cells. This would suggest that an endothelial cell-specific demethylase was responsible for demethylating the stably transfected, in vitro methylated eNOS promoter/reporter construct. The involvement of a cell-specific demethylase could be postulated. To date, a cell-specific demethylase has not yet been identified. However, an endothelial cell-specific factor may interact with a demethylase enzyme and thus achieve restricted expression of the eNOS gene through the DCR present on the eNOS promoter. Such experiments will allow the further dissection of the mechanisms controlling methylation to be defined. In addition, mutations of the positive regulatory domains (PRD I, II and III) may be used in these experiments to determine if promoter mutations will influence methylation status of the integrated methylated or non-methylated transfectants.

Future studies may also involve the investigation of methylation in the setting of diseased tissues. As discussed earlier, it is well established that endothelium-dependent vascular relaxation is perturbed in the setting of atherosclerosis and hypercholesterolemia. There is evidence to suggest that there is a loss of eNOS expression in endothelial cells over advanced atherosclerotic lesions. Perturbations in the methylation status of the human eNOS gene may represent a candidate molecular determinant of the atherosclerosis phenotype. To investigate this hypothesis, the methylation status of the human eNOS gene in normal versus atherosclerotic tissue will be assessed by the sodium bisulfite genomic sequencing method. Studying DNA methylation in the setting of diseased states provides a unique opportunity for defining novel aspects of such global regulatory mechanisms.
Our laboratory has developed promoter/reporter insertional transgenic mice lines containing the native murine eNOS promoter (-5200/+28) directing transcription of nuclear-localized β-galactosidase. Examination of β-galactosidase in the heart, lung, kidney, liver, spleen and brain of adult mice demonstrated robust signal in large and medium-sized blood vessels (304). This in vivo expression profile was uniform across multiple founders and not dependent upon the site of integration into mouse genomic DNA. In contrast, small arterioles, capillaries and venules were notable negative. Non-endothelial cells were positive only in the brain: the CA1 region of the hippocampus, Purkinje cells of the cerebellar cortex and outer cortical neuronal layers of the cerebrum. In summary, the findings of this work demonstrated that murine eNOS genomic regions spanning -5200/+28, relative to the transcription start site, direct expression of a reporter construct in a fashion that recapitulates the known expression profile of eNOS mRNA and protein. Future studies, therefore, may involve developing new insertional transgenes containing mutations or deletions of the original transgene. For example, a series of deletion promoter/reporter constructs could be generated and assessed in vivo in murine models. This will allow the mapping of promoter regions, which may play a role in the endothelial-specific expression of eNOS as well as the selective expression of eNOS in various endothelial cell types, such as large-sized vessels versus small arterioles or capillaries. In addition, linker-scanning mutations accounting for PRD I, II and III may be assessed in a similar approach. By virtue of the large degree of homology across species spanning these positive regulatory domains, the effect of mutating these important regulatory regions can be assessed in a murine model. Perhaps, a region of the promoter is responsible for the selective expression of β-galactosidase in different endothelial beds.
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