Characterization of the Human Endothelial Nitric-oxide Synthase Promoter*

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Fotula Karantzoulis-Fegaras‡, Hariclia Antoniou, Sheue-Lim M. Lai, Girish Kulkarni, Cheryl D’Abreo, Gordon K. T. Wong, Tricia L. Miller, Yvonne Chan, Judith Atkins, Yang Wang, and Philip A. Marsden§

From the Renal Division and Department of Medicine, St. Michael’s Hospital and University of Toronto, Toronto, Ontario M5S 1A8, Canada

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 study therefore 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 in vascular endothelium. Two 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 domains I (−104/−95 relative to transcription initiation) and II (−144/−115). Analysis of trans-factor binding and functional expression studies revealed a surprising degree of cooperativity and complexity. The nucleoprotein complexes that form upon these regions in endothelial cells contained Ets family members, Sp1, variants of Sp3, MAZ, and YY1. Functional domain studies in Dro sophila 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. Therefore, multiprotein complexes are formed on the activator recognition sites within this 50-base pair region of the human eNOS promoter in vascular endothelium.

Endothelial nitric-oxide synthase (eNOS) is the enzyme responsible, in major part, for endothelial-derived NO (1). 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 tone (2, 3), in remodeling of the vascular wall in response to changes in flow or distending hydrostatic pressure and in modulation of hemostatic pathways (4). 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.

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 implicated in signal transduction known as plasmalemal caveolae (5). N-Myristoylation and palmitoylation are necessary for efficient targeting and membrane insertion (6). Increases in intracellular calcium following endothelial activation facilitate interactions between the eNOS apoenzyme and calmodulin. This enhances NADPH-dependent electron flux through eNOS dimers resulting in the 5-electron oxidation of L-arginine and release of NO (7). Surprisingly, the molecular chaperone Hsp90 is also recruited to eNOS following cellular activation.

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 (8). Lysophosphatidylcholine (9), shear-stress (10–12), and transforming growth factor-β (13) represent important examples of exogenous stimuli known to modify eNOS gene transcription. An intriguing facet of the control of steady-state eNOS mRNA expression in vascular endothelium is the contribution of post-transcriptional regulation (14–17). The eNOS mRNA transcript normally has a very long half-life in vascular endothelium, usually greater than 24–48 h. Tumor necrosis factor-α (14, 18), hypoxia (15), entry into the cell cycle (16), and oxidized low density lipoprotein (17) 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.

In situ cRNA hybridization studies performed in a wide variety of human tissues revealed that eNOS mRNA transcripts are relatively endothelial cell-specific (8, 18). 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 ex-
pression of eNOS mRNA transcripts have been noted: syncytiotrophoblast of human placental villi, pyramidal cells of the CA1 region of the brain, and cardiac myocytes, among others. When compared with other genes expressed in vascular endothelium such as preproendothelin-1, endothelin converting enzyme-1, CD31/PECAM, or von Willebrand 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 Flik-1/KDR, Flt-1, Tie-1, Tie-2/Tek, and the cytokine-inducible adhesion molecule E-selectin (19). 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.

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 (20). 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 (21), also has functional importance in the transcription of other endothelial genes such as human von Willebrand factor, P-selectin, VCAM-1, and ICAM-2. However, given the broad tissue distribution of the GATA-2, 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, Flik-1/KDR, and Tie-2. cAMP response element and Ets motifs cooperate in activating the Flt-1 promoter (22). Three regions have been identified within the 5′-flanking sequences of the Flik-1/KDR gene containing putative Sp1, AP-2, NF-xB, and E-box elements important for functional activity in endothelial cells (23). More recent studies with the Tie-2 gene (24) identified negative (region I) and positive regulatory elements (regions U and A), although the trans-factors remain to be examined (24). 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.

Recently, this laboratory and others reported the isolation and characterization of complementary and genomic clones for eNOS (18, 25, 26). The human eNOS gene is present as a single copy in the haploid human genome and has been localized to 7q35–36 (26, 27). Structural characterization of genomic DNA revealed that the 4052-nt mRNA is derived from 26 exons distributed over 21 kilobases of human genomic DNA (26, 27). Even though analysis of 5′-flanking regions failed to define a canonical TATAA motif a single major transcription initiation site was defined by primer extension, S1 nuclease protection, and 5′-RACE to be 22 nt upstream of the translational start site (26). The early characterization of human eNOS genomic clones and 5′-flanking regions, by us and others, suggested a trivial model. Studies suggested a prominent role for Sp1 and GATA cis-elements in constitutive transcription initiation (26–29).

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. Two tightly clustered activator regions were identified in proximal enhancer regions 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) and PRD II (−144/−115). Analysis of trans-factor binding and functional expression studies revealed a surprising degree of cooperativity and complexity. 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, Eif-1, MAZ, and YY1.

### MATERIALS AND METHODS

#### Cell Culture—Bovine aortic endothelial cells (BAEC) and human umbilical vein endothelial cells (HUVEC) were isolated and characterized as described previously (16, 18). Schneider’s Drosophila line 2 (SL2) were obtained from ATCC (Rockport, MD), propagated in Schneider’s Drosophila medium supplemented with 10% fetal bovine serum and maintained at 23 °C with atmospheric CO2. Cell culture reagents were obtained from Life Technologies, Inc.

#### Promoter/Reporter Constructs—Restriction and modifying enzymes were from New England Biolabs (NEB) (Beverly, MA), Boehringer Mannheim (Mannheim, Germany), and Pharmacia (Uppsala, Sweden).

#### Plasmid DNA was prepared using two rounds of gradient-sedimentation ultracentrifugation in ethidium bromide-saturated cesium chloride cushions. Multiple independent preparations were employed in transfection experiments. Comparable findings were evident when plasmid DNA was prepared in dam-, dcm- bacteria.

#### Deletion Mutants—An EMBL3 phage clone containing human eNOS genomic sequences was isolated using Southern blot hybridization (26). A 3.6-kb Apal fragment, extending from −3500 to +113 relative to the major transcription start site, was subcloned into the Apal site of pBluescript SK I (−) (Stratagene, La Jolla, CA). The 3.6-kb Apal fragment was isolated by preparative gel electrophoresis, blunted-ended with Klenow enzyme, and ligated to 12-bp SauI linkers (NEB). The 3.6-kb SauI fragment was then subcloned into the Xhol site of the promoterless reporter construct, pGL2-Basic (Promega, Madison, WI), designated as pGL2−3500/+109. pGL2−3500/+109 was used as a template to generate pGL2−1900/+109, pGL2−1193/+109, pGL2−1001/+109, pGL2−743/+109, pGL2−265/+109, pGL2−49/+109, and pGL2−14/+109 by means of restriction enzyme digestion. The integrity of promoter/reporter gene constructs was assessed with DNA sequence analysis. PCR was used to generate pGL2−217/+109, pGL2−185/+151 to +109, pGL2−153/+109, and pGL2−104/+109. Oligonucleotides were synthesized using a Beckman Oligo 1000 DNA synthesizer (Beckman Instruments, Fullerton, CA). Constructs generated with PCR were sequenced using an automated ABI Prism 377 DNA sequencer (Perkin-Elmer Applied Biosystems Canada Inc., Mississauga, ON) to monitor for PCR-associated nucleotide incorporation errors.

−1193/+109 Linker Mutants—Twelve linker-scanning mutations, containing the sequence 5′-GGCAGATCCGC-3′, and spanning a 120-bp region of the human eNOS promoter from −164 to −45, relative to the transcription start site, were created. These mutations were incorporated into the pGL2−1193/+109 construct. Two types of ampicils, namely an A amplicon (the 5′-fragment) and a B amplicon (the 3′-fragment), were created using a modified PCR-based method. Primers used to create the linker-scanning mutants are listed in Table I. The A amplicon was generated using two primers: Amin and An. Amin is a gene-specific sense primer that is homologous to −327 to −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: Bmin and Bn. Bn is a 25-mer, sense primer containing 15 bp of homologous eNOS sequence and a heterologous 10-bp mutation at the 5′-end of the primer. Bmin is a 21-mer homologous to the luciferase reporter gene sequence in the pGL2-Basic vector. A and B amplicons were subcloned into the pCR II vector (Invitrogen Corp., San Diego, CA) and sequenced. The A and B
amplicons were subsequently subcloned between the PstI and BglII sites of the −1193/+109 pGL2 promoter/reporter luciferase construct. −265/+109 Linker Mutants—Each pGL2 −1193/+109 linker mutant construct was digested with SmaI (a 3′-multiplying clone site within pGL2-Basic vector) and PstI (−265), blunt-ended with Klenow enzyme, and ligated.

**Drosophila Eukaryotic Expression Constructs**—Expression cassettes for Sp1, Sp3 variants, Elf-1, Ets-1, and MAZ were based upon pPacU, a transient episomal vector which contains the 2.6-kb *Drosophila* actin 5C promoter, a 0.7-kb 5′-UTR Ultratrichor (UxI) internal ribosome entry site, the first eight codons of the Ubx open reading frame and 1.1-kb 3′-UTR from the actin 5C gene. The latter provides polyadenylation signal sequences. pPacUSp1 and pPacUO was kindly provided by Thomas Shenk (Howard Hughes Medical Institute, Princeton, NJ) (33).

BglII plasmid was subjected to a partial digestion with the restriction enzymes NdeI and BamHI. A 1.9-kb XhoI fragment was subcloned into the pPacUSp3 main 5′ promoter, a 0.7-kb 5′-UTR from the actin 5C gene. The latter provides polyadenylation signal sequences. pPacUSp1 and pPacUO was kindly provided by J. M. Leiden (University of Chicago, Chicago, IL). For construction of pPacUSp3, human eNOS Promoter

### Table I

| Primer set Region | Primer set |
|-------------------|------------|
| 5′-GGGAGGGTGAAGGAAGAAGAGCC-3′ | −496 to −305 |
| 5′-GACCCCAACAGGGGAGC-3′ | −347 to −114 |
| 5′-CACACTTACACATACAGGAAAC-3′ | −220 to +19 |
| 5′-AGCTTGTGGCTCAGTCTCC-3′ | −7 to +171 |

**Luciferase Assays**—48 h after transfection, cells were harvested with 300 μl of lysis buffer (0.1 M potassium phosphate buffer (pH 7.8), 1% Triton X-100, 1 mM DTT, 2 mM EDTA). Protein extracts were centrifuged at 10,000 × g for 2 min to pellet residual cellular debris and stored at −80°C for subsequent assay. The Bio-Rad protein assay was used to determine protein concentration using bovine serum albumin as a reference standard. Reverse transcription-PCR was used to characterize RNA species produced from transfected DNA constructs to confirm that episomal eNOS promoter/reporter constructs utilized comparable sites for transcription initiation as the native promoter (data not shown). Luciferase assays were carried out on the Monolight 2010 luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI). Coenzyme A and ATP were from Calbiochem (La Jolla, CA). t-Luciferin and CPRG were purchased from Molecular Luminescence Laboratory. Briefly, 35 μl of protein extract, 100 μl of t-luciferin (1 mM), 100 μl of luciferase assay buffer (30 mM Tricine (Calbiochem, La Jolla, CA), 3 mM ATP, 15 mM MgSO4, 10 mM DTT, 1 mM coenzyme A), and a pH of 7.8 were used. Measurements of light units were integrated over a 10-s interval. Data in raw luciferase units (RLU) were normalized for the nonspecific background of mock-transfected cells, which represented 0.5% of maximum experimental luciferase activities. Intra- and inter-assay coefficients of variation averaged 7% and 9%, respectively.

**β-Galactosidase Assay**—CPRG (chlorophenol red-β-D-galactopyranoside, Boehringer Mannheim) was used as the chromogenic substrate for β-D-galactosidase measurements. 40 μl of cell extract was mixed with 180 μl of assay reagent containing 70 mM sodium phosphate buffer (pH 7.3), 7.3 mM MgCl2, 8 mM CPRG solution, and 0.1 mM 2-mercaptoethanol. CPRG was made fresh for each assay in 0.1 M sodium phosphate buffer (pH 7.3). Reaction mixtures were incubated at 37°C for 20 min and subsequently stopped with 500 μl of 1 M Na2CO3. Enzymatic activity was measured at 570 nm. As a index of the endogenous β-galactosidase activity, 40 μl of mock-transfected cell extract was used and for BAEC averaged 1% of raw values. As a positive control, 1 μl of β-galactosidase (Sigma, 50 units/ml) was added to 40 μl of mock-transfected cell extract.

**Single-stranded Conformation Polymorphism Analysis of eNOS Promoter Regions**—A focused SSCE screen of the core promoter consisted of four overlapping ampiclons, averaging 180 nt in size, extending from −456 to +133 (Table II). Peripheral blood genomic DNA was obtained from 25 healthy, unrelated, and ethnically diverse individuals using standard methodology after informed consent. PCR reactions were carried out with the following conditions: 1 cycle of 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 10 min.
ried out in 25-μl reaction volumes with 25 ng of peripheral blood genomic DNA, 12.5 pmol of each primer, 1.25 units of Taq polymerase (Life Technologies, Inc.), 100 μM of each dNTP, 1 μCi of [α-32P]dCTP (NEN Life Science Products), 0.75–1.0 mM MgCl2, 20 μg of Tris-HCl, pH 8.4, and 50 mM KCl. A “hot start” was carried out with the addition of [α-32P]dCTP and dNTP after an initial denaturation at 94 °C for 5 min and was followed by 30 cycles of 94 °C for 30 s, optimal annealing temperature for 30 s, and a 72 °C extension for 30 s, followed by a single final extension at 72 °C for 15 min. At the completion of thermocycling, 7 μl of the PCR product was mixed with 5 μl of loading buffer (95% formamide, 10 mM NaOH, 0.25% bromphenol blue, 0.25% xylene cyanol, 300 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride) and incubated on ice for 15 min. To disrupt cytoplasmic membranes, size-fractionation was carried out using trypsin-EDTA, washed twice with PBS at 4 °C, and resuspended gently in 400 μl of ice-cold 10% Nonidet P-40 was added and the mixture was subjected to centrifugation (10,000 g, 80 °C with intensifying screen. Monoclonal and polyclonal antibodies were from Santa Cruz Biotechnology: Sp1 (PEP2), Sp2 (K-20), Sp3 (D-20), Sp4 (K-20), YY-1 (H-414), GATA-2 (CG2–96), Ets-1 (NH2 terminus, N-276), Ets-1 (COOH terminus, C-20), Ets-2 (C-20), PU.1 (Sp1-1, T-21), Erg-1 (C-17), Fli-1 (C-19), PEA3 (16), and Elk-1 (I-20). Anti-Elk-1 (rabbit polyclonal), anti-MAZ (murine monoclonal), and anti-GATA-2 (rabbit polyclonal) were generous gifts from J. M. Leiden (University of Chicago, Chicago, IL), R. B. Marcus (State University of New York, Stony Brook, NY), and S. H. Orkin (Harvard Medical School, Boston, MA), respectively.

**RESULTS**

**Functional Analysis of Human eNOS 5′-Flanking Regions**—To analyze the mechanisms of transcriptional regulation important in the human eNOS gene and to define functionally important cis-DNA elements in the 5′-flanking region of this gene, 13 eNOS 5′- to 3′-deletion promoter/reporter constructs were generated. All had a variable 5′-end but a common 3′-end, ending at +109 relative to the start site of transcription. Luciferase expression was assayed following transient transfections of BAEC or HUVEC and normalized using β-galactosidase activity and protein concentrations. As shown in Fig. 1A, the construct pGL2−1193/+109 evidenced maximal functional eNOS promoter activity in BAEC, and averaged ± 1% (mean ± S.E.) of the activity of the SV40 promoter/enhancer (n = 3, triplicate determinations). Characterization of RNA species transcribed from transfected DNA constructs with reverse transcription-PCR confirmed that epo-somal eNOS promoter/reporter constructs utilized sites for transcription initiation that were comparative to the chromatin transcription unit (data not shown). The constructs pGL2−3500/+109 and pGL2−1900/+109, which contain further 5′-flanking sequences, had moderately lower activities than pGL2−1193/+109 (70% and 80%, respectively, of the activity of pGL2−1193/+109), suggesting the presence of cis-regulatory DNA sequences exhibiting negative functional effects between −3500 and −1193. Deletion of sequences from −1193 to −1001 produced a ∼20% decrease in activity. Removal of sequences spanning −1001 to −265 did not produce an appreciable drop in functional promoter activity. When sequences from −265 to −14 were deleted, a marked drop in activity was evident. Values for the −14/+109 construct essentially reflected those seen for the promoterless luciferase plasmid, pGL2-Basic.

A further series of transient transfections were designed to examine the regions between −265 and −14 in greater detail (Fig. 1B) (n = 3, triplicate determinations). Constructs with 5′-end points at −265, −217, −185 and −151 demonstrated comparable activity that represented approximately 60–70% of

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**Table III**

| Oligonucleotide used in EMSA analysis | Sequence (5′ to 3′; sense strand) |
|--------------------------------------|----------------------------------|
| −120/−91                            | CAT TGT GTA TGG CAT AGG GGC GGG AGG |
| −120/−91 mutSp1                     | CAT TGT GTA TGG CAT AGG GGC GGG AGG |
| −120/−91 mutGATA                    | CAT TGT GTA TGG CAT AGG GGC GGG AGG |
| Sp1 (SV40 early promoter)            | ATT CGA TGG CGG GGG GGG GAG |
| Ap2 (hMIFa promoter)                | CAT TGT GTA TGG CAT AGG GGC GGG AGG |
| −140/−111                           | TCC CAG CGG GGC TGC TGT CTC CAT TGT GTA T |
| −155/−120                           | GCT TTA GAC CCT CCC AGC GCG GCT TGT TGG TCC |
| HIV-2 CD3R                          | TCG AGT TAA AGA CAG GAA CAG CTA TGT CAG |
| HTLV-1LTR                           | TCG AGG GGA GGA AAT GGG TGT CCA |
| PEA3                                 | TCG AGG AGG AAG TGA TGT CGT |
| SRY-AM                              | TCG AGG AGG AAG CAT TGT GGC TGC |
| p53mut1                             | AGC TTT GCC TGG ACT TGC CTG GCC TGT CCC |
| p53mut2                             | AGC TTT GGC TGG ACT TGC CTG GGC TGT TCC |
| PAX-2                                | AGC ACC GTT CCG CTC ACC GCT CTT CAC ACC GTT CCG CTC |
| PAX-8                                | CTA AGC TTG AGG CAT CAG AGC AGG AGC AGG GC |
| YY1                                  | TGC CTT GCA AAA TGG CGT TAC TGC AG |

* Underlined sequence refers to mutated nucleotides.
the most active construct, pGL2−1193/+109. Deletion of sequences between −151 and −133 produced an additional 33% drop in activity and removal of the sequences from −133 to −92 abrogated remaining functional promoter activity of the eNOS 5′-flanking region. Taken together, these findings suggest that two positive regulatory regions exist in the proximal core promoter of the human eNOS gene, the first between −151 and −133, and the second between −133 and −92. Comparison of
the functional promoter activity of a series of human eNOS promoter/reporter constructs in HUVEC (Fig. 1C) 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.

To gain a more thorough understanding of the mechanisms implicated in the regulated expression of the eNOS gene in endothelial cells, a systematic and comprehensive mutational analysis of eNOS proximal promoter regions was conducted by mutating consecutive 10-bp sequences across the 5′-flanking region. All mutants contained a 10-bp sequence not known to contain functionally important cis-regulatory elements (5′-GCAGATCCGC-3′). Such studies allow functionally important regions downstream of −133 to be defined and allow transcriptional regulation of eNOS to be analyzed while maintaining the native topological and spatial relationships of the DNA helix. Twelve 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. As shown in Fig. 2A, four of the eNOS promoter/reporter linker-scanning mutants demonstrated significantly decreased functional activity in BAEC compared with wild-type sequences (n = 4, triplicate determinations). 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). In addition, the 10-bp mutation spanning −104/−95 (−104/−95mut) resulted in a profound 85% drop in luciferase activity (p < 0.0001).

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 of the shorter promoter linker mutants with the activity profile of the same 10-bp mutations in the context of a longer eNOS promoter region allowed large domain interactions to be defined and/or mapped. Functional reporter activities revealed no important differences compared with linker-scanning mutations in the setting of the maximally active −1193/+109 construct. A synopsis of these data is presented in Fig. 2B and indicated that functional contributions from PRD I and PRD II are not dependent upon additional sequences present between −1193 and −265 (n = 3, triplicate determinations). Although 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). Taken together as a whole, deletion and linker-scanning functional studies defined two regions of interest: −104 to −95 (designated as PRD I) and −144 to −115 (PRD II).

Functional characterization of the proximal eNOS promoter utilized cis-DNA sequences previously reported by this, and other, laboratories (26, 27). To determine if the reported findings were more broadly applicable, SSCP was utilized to determine whether any common allelic variants exist in a healthy general population. Characterization of eNOS sequences spanning −496 to +171 in 50 chromosomes (25 peripheral blood genomic samples, four overlapping SSCP amplicons) revealed no electrophoretic SSCP variants. This suggested that there were no common allelic variants for human eNOS genomic regions internal to the SSCP primers (−475 to +152) and that findings relevant to the current work were broadly applicable (data not shown).

Nucleoprotein Complexes Formed by PRD I—A series of protein-DNA EMSA complexes were observed with the labeled −140/−111 probe and BAEC nuclear extracts (Fig. 4A, 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 (Fig. 4A, lane 4). Competition studies performed with unlabeled −120/−91 probe and a heterologous consensus Sp1 high affinity site from the early SV40 promoter indicated comparable effectiveness in competition studies (data not shown).

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 (Fig. 4A, 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 (Fig. 4A, lane 4). Competition studies performed with unlabeled −120/−91 probe and a heterologous consensus Sp1 high affinity site from the early SV40 promoter indicated comparable effectiveness in competition studies (data not shown).
FIG. 2. Activity profiles of human eNOS promoter/reporter luciferase linker-scanning mutations in BAEC. A, linker-scanning mutants introduced a 10-bp substitution (5-GCAGATCCGC-3'). B, comparison of promoter activity of PRD I and II mutant pGL2-1193/+109 and pGL2-265/+109 constructs. 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 percent luciferase activity relative to pGL2-1193/+109 and represent the mean ± S.E. (three independent experiments, triplicate determinations). The maximally active construct, pGL2-1193/+109, displayed 10–20% of the activity of SV40 promoter/enhancer-directed luciferase control vector, pGL2-control. Where error bars are not evident, S.E. is below the figure resolution.
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 (47–49).

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 (Fig. 4B, lane 5). These findings imply that the fast migrating complex contains an Elf-1-like protein is present in the BAEC 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 consensus DNA sequences evident in PRD II (−120/−91) (36). 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 (36) (Fig. 4A, lane 4). A clear reduction in one of the fastest migrating bands resulted in a supershift of the fastest migrating complex (Fig. 4A, 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.

MAZ (for myc-associated zinc finger) is a zinc-finger transcription factor that displays protean roles in transcription initiation, interference, and termination (39). MAZ was originally identified in the c-myc P2 promoter and is known, through binding site sequence assays, to bind to GGAGGG- or CCGTCCC (CT elements) (39, 40). 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 (51, 52). 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 (33). 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 competition for some, but not all of the complexes (Fig. 5A, 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 (Fig. 5B, 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 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 (Fig. 5A, lane 5).
FIG. 4. EMSAs of BAEC nuclear protein binding to −140/−111 PRD II eNOS 5′-flanking region. A, lane 1 represents probe alone. Lanes 2 and 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: unlabeled −140/−111 (lane 4), HIV-2 CD3R (lane 5), HTLV-I LTR (lane 6), STROM (lane 7), and PEA3 (lane 8) (2.5 × 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 unlabeled −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 were 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 × 10^4 dpm labeled probe), and 10 µg of BAEC nuclear extract was added in lane 2. 100-fold molar excess of unlabeled 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.

FIG. 5. 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 unlabeled −155/−120 and −140/−111 probes were added in lanes 3 and 4, respectively (2.5 × 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-Sp1 (lane 3), anti-Sp3 (lane 4), and anti-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.
in shift abrogation of complexes formed upon the −155/-120 probe, especially one of the prominent fastest migrating ones (Fig. 5B, 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 (Fig. 4C, 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.

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 (Fig. 2A). Similarly, mutating the region encompassing an Ets recognition sequence (−129/-126) also resulted in a reduction of promoter activity (Fig. 2A). 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 that lack constitutive Sp1, Sp3, and Ets activities, namely the Drosophila Schneider cell line (for each experimental series, n = 3, triplicate determinations) (31, 32). Ets-1 is a well characterized member of the Ets family of transcription factors that is known to be robustly expressed in endothelial cells (53, 54). 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 Fig. 6A, cotransfection of increasing amounts of Sp1 expression cassette, over the range 1–250 ng/plate, resulted in a concentration-dependent increase in functional promoter activity (n = 3, triplicate determinations). 250 ng of Sp1 expression plasmid resulted in a maximal 140-fold increase. Increasing amounts of expression cassette encoding full-length Sp3, over the range 1–250 ng/plate, also stimulated luciferase activity in a concentration-dependent fashion, with a maximal 35-fold increase (Fig. 6A). Co-expression of Sp3 with half-maximal amounts of Sp1 (15–40 ng) demonstrated cooperative positive functional interaction with the eNOS promoter (Fig. 6A). Transferring increasing amounts of Ets-1 expression plasmid alone failed to modify functional eNOS promoter activity over the range 1–250 ng/dish (Fig. 6B). However, co-expression of Ets-1 with half-maximal amounts of Sp1 resulted in a cooperative positive functional interaction with the eNOS promoter (Fig. 6B) (n = 3, triplicate determinations). Although threshold amounts of Ets-1, Sp3, and Sp1 expression cassettes (5 ng) alone had minimal effects on the pGL2−1193/+109 luciferase reporter construct (Fig. 6C) (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 with 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 three 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 (Fig. 6C). 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 (Fig. 6C). 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 Exhibits a Negative Effect on eNOS Functional Promoter Activity—MAZ can both activate and inhibit transcription initiation (39). The functional contribution of MAZ to eNOS promoter/reporter was assessed in Drosophila Schneider cells using co-transfection experiments. Cotransfection of increasing amounts of MAZ expression plasmid alone, over the range 1–250 ng/plate, did not have any effect on activity of the −1193/+109 eNOS promoter/reporter luciferase construct (Fig. 7A) (n = 3, triplicate determinations). An additional 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 (Fig. 7B) (n = 3, triplicate determinations). This suggested that MAZ has a negative effect on the cooperative interaction among Ets-1, Sp3, and Sp1. MAZ had a similar effect on Sp1 alone (Fig. 7C) (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 with Sp1 alone. Various linker-scanning mutant constructs were used to define the requirements for PRD I and PRD II cis-elements (Fig. 7D) (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. Although base-line 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 were 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 repression activity is especially dependent on the Ets site in PRD II.

Functional Studies Evaluating the Effect of NH2-terminal Deleted Sp3 in Drosophila Schneider Cells—In mammalian cells the nucleoprotein Sp3 exists as 3 protein isoforms as a result of alternate usage of translational initiation sites (55). One isoform with an apparent molecular mass of 110 kDa represents the full-length Sp3 protein, whereas the other two isoforms with apparent molecular masses 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 (55). 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 (55). Given the above findings that EMSA using endo-
FIG. 6. Sp1, Sp3, and Ets-1 transactivate human eNOS promoter/reporter luciferase constructs in Drosophila Schneider cells. A, assay of promoter activity of pGL2-1193/+109 promoter/reporter luciferase construct upon co-transfection with increasing amounts of pPacUSp1 (5–250 ng, left panel), pPacUSp3 (5–250 ng, middle panel), and pPacUSp3 (5–250 ng) with half-maximal amounts of pPacUSp1 (40 ng) (right panel). B, assay of promoter activity of pGL2-1193/+109 promoter/reporter luciferase construct upon co-transfection with increasing amounts of pPacUSp1 (5–250 ng, left panel), pPacUEts-1 (5–250 ng, middle panel), and pPacUEts-1 (5–250 ng) with half-maximal amounts of pPacUSp1 (40 ng) (right panel).
thelial 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 Fig. 8A (n = 3, triplicate determinations), NH2-terminal deleted Sp3 expression cassette (Sp3\textsuperscript{D}NH2) was no longer capable of trans-activating the promoter by itself over the range 1–250 ng/plate (compare with Sp3 in Fig. 6A). Sp3\textsuperscript{D}NH2, however, dramatically enhanced the cooperative activity exhibited by threshold amounts of Ets-1, Sp3, and Sp1, at both 5 or 100 ng of Sp3\textsuperscript{D}NH2 (Fig. 8B) (n = 3, triplicate determinations). On the other hand, when varied amounts of Sp3\textsuperscript{D}NH2 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 (Fig. 8C) (n = 3, triplicate determinations). A low amount of Sp3\textsuperscript{D}NH2 (1 ng) exhibited a repressive effect on Sp1-mediated activation, whereas cooperativity was demonstrated between Sp1 and higher amounts of Sp3\textsuperscript{D}NH2 (250 ng). Low amounts of Sp3\textsuperscript{D}NH2 was also able to repress Sp3-mediated activation (100 ng) of the eNOS promoter in Drosophila Schneider cells (data not shown).

**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 1–250 ng/plate, minimally enhanced eNOS promoter activity (Fig. 9A) (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) (Fig. 9B) (n = 3, triplicate determinations). In other words, Elf-1 exerted a negative effect on the ability of Sp1 protein to activate the

![Figure 7](image_url)

**FIG. 7.** Effect of MAZ on eNOS promoter activity in *Drosophila* Schneider cells. **A**, promoter activity of pGL2 –1193/+109 construct following co-transfection with increasing amounts of pPacUMAZ (5–250 ng). **B**, 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). **C**, MAZ (pPacUMAZ, 100 ng) inhibits pGL2 –1193/+109 promoter/reporter luciferase construct activity in the presence of half-maximal amounts of pPacUSp1 (15 ng). **D**, 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 three times. Data are expressed as -fold increase in luciferase activity ± S.E. relative to pGL2 –1193/+109.
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/Sp1 expression plasmids (Fig. 9C) (n = 3, triplicate determinations). Conversely, cytomegalovirus-directed expression of Elf-1 protein in BAEC had a stimulatory effect on the −1193/+109 eNOS promoter/reporter construct (Fig. 9D). 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 3.2–13.6-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 with the wild-type −1193/+109 eNOS promoter/reporter construct (data not shown).

**DISCUSSION**

Two tightly clustered activator regions were identified in proximal regions of the human eNOS promoter using deletion analysis and linker-scanning mutagenesis: PRD I (−104/−95) and PRD II (−144/−115). 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 (Fig. 1). As well, there is a high degree of relatedness in human and bovine genomic DNA sequences for PRD I and PRD II (56), and no common allelic variants in these genomic regions for human eNOS were detected (−475 to +152).

Sp1, Sp2, Sp3, and Sp4 are closely related members of a gene family encoding zinc-finger (His,Cys) 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 (32). 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 (Fig. 10). 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 (57). This contrasts with other promoters, such as the HIV-1 LTR, wherein Sp3 acts as an inhibitor of Sp1-mediated activation (58). When Sp1 and Sp3 were expressed concomitantly in cells, Sp3 inhibited Sp1-mediated activation of the HIV-1 LTR (32). 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 up-stream of the DNA-binding domain (57).

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 (55). 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 are recognized by antisera prepared against the Sp3 protein (55). 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. (55) concluded that the internally initiated Sp3 isoforms function as potent inhibitors of Sp-mediated tran-scription, 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 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 transcrip-tion depending on the number of Sp1/Sp3 binding sites in the promoter (55). 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 co-ordinated interactions created through protein-protein and protein-DNA interactions

![Diagram](image-url)
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 p21Cip1/WAF1 expression upon keratinocyte differentiation (59). 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 (60).

Studies in endothelial and Drosophila Schneider cells indicate a critical role for PRD II in efficient transactivation of the eNOS promoter. Although 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 putative cis-DNA elements (Fig. 10). 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 (61). 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, Ets-2, and Erg-1 protein (53, 62). Angiogenesis and cytokine activation represent important changes in endothelial phenotype that are associated with alterations in Ets family member expression and function (53, 63).

Given EMSA results (Figs. 4 and 5) 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 (Fig. 6). 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 (Fig. 6C). 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 (64). Similarly, the P4 promoter of parvovirus minute virus is synergistically transactivated via neighboring Ets-1 and low affinity Sp1 sites (65). 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

**Fig. 10.** Schemata representing the proximal promoter of the human eNOS gene. PRD I and II are indicated. Functional cis-regulatory DNA elements are shown. The positions of three EMSA oligonucleotides (−155/−120, −140/−111, and −120/−91) are shown as solid black lines. Numbering is with respect to the start site of transcription (arrow).
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 (47). 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 (47). This concept is relevant to a model discussed below.

With the exception of GABPα, 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′-ACAG-GAACAA-3′) with previously identified Ets binding sites revealed near identity with the HIV-2LTR enhancer (5′-ACAGGAAAGC-3′), which is known to bind Elf-1 (50) (Table IV). Elf-1 is a transcription factor previously implicated in the 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 interleukin-2 receptor α chain (IL-2Rα) following T cell activation (51, 66). Elf-1 plays a role in the developmental regulation of the terminal transerase gene in early lymphocyte development (67). Elf-1 is also required for inducible T cell trophic viruses including HIV-2 and HTLV-I (50, 68). Elf-1 is highly expressed in B cells, where it participates in the regulation of a variety of genes, including IgH, lyn, and ick (69). As a rule, the Ets family of transcription factors are 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 (61). For example, FÜ.1 is a B cell- and macrophage-restricted and ESE-1 is primarily found in epithelial cells (70). Elf-1 protein expression was thought to be relatively restricted to lymphoid and myeloid cells (66). The present work demonstrates that Elf-1 is constitutively expressed in vascular endothelium and can bind to PRD II and consensuses 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, Elf-2, and MEF were identified (69, 71). These proteins contain domains that are structurally similar to the Ets binding domain of Elf-1 (69, 71). 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)CCGGAAGT(a/g/c)-3′) determined by a binding site selection method does not conform to known functional Elf-1 sites (Table IV) (43). Conversion of a naturally occurring, low affinity Elf-1 site in the IL-2Ro promoter to an optimal site resulted in a decrease in the ability of Elf-1 to induce transcription (43). 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 (72). 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 T cell receptor-ζ 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 (73). 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 Elf-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 Elf-1 binding site in PRD II.

Table IV  Comparison of known Elf-1 binding sites

| eNOS (PRD II) | A | C | A | G | G | A | A | C | A |
| HIV-2 LTR | A | C | A | G | G | A | A | C | A |
| IL-2Ro | A | T | A | G | G | A | A | G | T |
| CD4 | A | C | A | G | G | A | A | G | T |
| GM-CSF | A | G | A | G | G | A | A | T |
| IL-3 | G | C | A | G | G | A | A | G | G |
| HTLV-I LTR (PuB1) | C | C | G | A | A | G | C |
| HTLV-I LTR (PuB2) | G | G | A | G | G | A | A | G | T |
| hTCR-ζ chain | C | A | G | G | A | A | G | T |
| Consensus derived from binding site selection | C/a | C | C | G | G | A | A | G | T |

Human eNOS Promoter

3091

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 (76). It may be possible that Elf-1 requires a co-activator in order to demonstrate activation potential. Also, activation of mitogen-activated protein kinase signal transduction pathways results in changes in the activity of many ETS domain transcription factors (77). 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 (78). 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 com-
plexes 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 (79). After T cell activation, phosphorylation of Rb leads to the release of transcriptionally competent Elf-1 (79). 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 (Fig. 4D), likely at position −121 to −117 (Fig. 10). YY1 is a ubiquitously expressed 65–68-kDa GLI-Kruppel-related protein that contains four C2H2-type zinc fingers at the COOH terminus (80) 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 (81), the oncoprotein c-Myc, cyclinH A, FK506-binding protein, p300, ATF/CREB, and the mammalian homologue of RPD3 (82). Given this complexity, future studies will be needed that address the functional contributions of YY1 to eNOS promoter structure and function in episonal 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 (81) 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 (GABPα) promoter (83, 84). 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 (83). 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 (85). 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 (86), given that YY1 has also been implicated in the formation of preinitiation transcription complexes independent of TATA-binding protein (87).

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 protein roles in gene regulation: transcription initiation, interference, and termination (39). MAZ is especially important in TATA-less promoters (33). 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, 39, 52). An important facet of MAZ function is the participation of partner proteins. MAZ polypeptide contains multiple functional domains in addition to the six structurally important zinc fingers (His2Cys2) (39). Functional interactions between MAZ and Sp1 occur in a number of genes, including c-myc (33, 40). In the case of CD4, enhancer activity during development is critically dependent upon MAZ and an Ets consensus site that binds Elf-1 (51, 52). 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 ME1a1 site in the P2 promoter, but MAZ and ME1a1-like binding sites are also involved in the transcriptional pausing/attenuation of the c-myc gene, and also the human complement C2 gene (39). 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. Although 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. Sp1, variants of Sp3, Ets-1, and Elf-1 play important roles in the activation of eNOS transcription in endothelial cells (Fig. 10). Cooperativity between these trans-acting factors is likely to require multiple protein-protein and protein-DNA interactions (88, 89). These trans-acting factors may 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 with 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-κB, and 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 (90) upon binding. These DNA deformations may be important in determining overall binding affinities as well as influencing binding site preferences for neighboring sites, but are not by themselves sufficient for transactivation (90). These changes may also enhance the transactivation potential of Sp1 family members. Ets factors also interact with other proteins to form either multisubunit 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 further 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 (80) indicate that protein-DNA and protein-protein
interactions on the eNOS promoter may be modulated by complex architectural features. In this regard, further detailed biochemical analysis of protein-DNA and protein-protein interactions on the eNOS promoter will be needed to substantiate this model further. 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 athero-clerosis.

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