Sequences in Intron 51 of the von Willebrand Factor Gene Target Promoter Activation to a Subset of Lung Endothelial Cells in Transgenic Mice

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In vivo analyses of the VWF promoter previously demonstrated that a fragment spanning sequences −487 to +247 targets promoter activation to brain vascular endothelial cells, whereas a longer fragment including 2182 bp of the 5’-flanking sequences, the first exon, and the first intron activated expression in endothelial cells of the heart and muscles as well as the brain of transgenic mice. These results suggested that additional VWF gene sequences were required for expression in other vascular endothelial cells in vivo. We have now identified a region within intron 51 of the VWF gene that is DNase I-hypersensitive (HSS) specifically in nonendothelial cells and interacts with endothelial and nonendothelial specific complexes that contain YY1. We demonstrate that β-actin is associated with YY1 specifically in the nucleus of nonendothelial cells and is a component of the nuclear protein complexes that interact with the DNase I-hypersensitive region. In vitro transfection analyses demonstrated that HSS sequences containing this YY1-binding site do not significantly affect VWF promoter activity. However, in vivo analyses demonstrated that addition of these sequences to the VWF promoter (−487 to +247) results in promoter activation in lung and brain vascular endothelial cells. These results demonstrate that the HSS sequences in intron 51 of the VWF gene contain cis-acting elements that are necessary for the VWF gene transcription in a subset of lung endothelial cells in vivo.

von Willebrand factor (VWF)2 is an adhesive protein involved in healing wounds of the vasculature (1). The VWF gene, located on chromosome 12, is 178 kb long and contains 52 exons (1, 2). VWF is synthesized exclusively by endothelial cells and megakaryocytes. We have previously characterized a region of the VWF gene spanning sequences −487 to +247 that functions as an endothelial specific promoter in vitro. Trans-acting factors NF1, Oct 1, Ets, GATA6, HLP, NF-Y, and Ebp4 that positively and negatively regulate the activity of this promoter fragment were identified by others and us (3–10). However, in vivo analyses of this promoter region in transgenic mice demonstrated that it targets the expression of a fused LacZ gene to a subset of brain vascular endothelial cells in adult transgenic mice (11). Additional sequences of the VWF gene that extended the 5’ region of the promoter to −2182 and the 3’ end of the promoter to the end of the first intron were shown to activate transcription in endothelial cells of the heart and muscles as well as the brain of transgenic mice (12). These results suggest that distinct regions of the VWF gene are required to achieve promoter activity in the endothelial cells of distinct organs.

To identify additional cis-acting elements within the VWF gene that may participate in transcriptional regulation, we explored the possibility that such sequences may be located in VWF chromatin regions that show hypersensitivity to DNase I. Many transcriptional regulatory elements are located within chromatin regions hypersensitive to DNase I and other nucleases. These hypersensitive regions usually contain binding sites for one or more transcription factors that can be either common or tissue-specific (13). Using this approach we identified a region within intron 51 of the VWF gene that interacts with YY1-containing nuclear protein complexes and targets VWF promoter activation to a subset of lung vascular endothelial cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—Cell culture of HeLa and human umbilical vein endothelial cells (HUVECs) were carried out as previously described (5). Human primary fibroblasts (HUMFIB) were maintained as described for HeLa cells. Sheep pulmonary artery endothelial cells were prepared as previously described (14) and maintained in Opti-MEM (Invitrogen) with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C with 5% CO2. Transfections were performed using the Lipofectamine Plus system as described by the manufacturer (Invitrogen).
Preparation of Nuclei, DNase I Digestion, and Southern Blot Analyses—Nuclei were prepared using a modification of a previously described procedure (15). Aliquots of nuclei containing 2 \( A_{260} \) units were treated with 0–15 units/ml RNase I (Promega) for 10 min at 37 °C, and the reactions were quenched by addition of an equal volume of 25 mM EDTA, 2% SDS, and subjected to 200 \( \mu \)g/ml proteinase K digestion followed by RNase treatment. Isolated DNA was digested to completion with EcoRI and subjected to Southern blot analyses as described (16). The probe for Southern blot analyses was synthesized using polymerase chain reaction with plasmid 2.3\( \lambda \) containing EcoRI fragment 38 of the VWF gene (2) (gift of J. E. Sadler) as template and the following primers: 5’-GGGACCTATTTCCAGCCCGAATGAG-3’ (forward) and 5’-CCCTCCGCACCCAGGCCCTATTG-3’ (reverse).

Nuclear Extract Preparation, Electrophoretic Mobility Shift Analysis, Immunoprecipitation, and Western Blot Analyses—Nuclear extracts preparation, gel mobility, and supershift assays were carried out as previously described (4, 5). Immunoprecipitation and Western blot analyses were performed as previously described (17). Antibodies used were mouse monoclonal anti-\( \beta \)-actin clone AC-15 (Sigma) and rabbit polyclonal anti-YY1 (Santa Cruz Biotechnology H-414). Mass spectrometry analyses were performed on the Coomassie Blue-stained polypeptide sliced from the gel using liquid chromatography-tandem mass spectrometry analyses by University of Alberta Peptide Institute as previously described (10). Tandem mass spectrometry spectra were directly used to search the National Center for Biotechnology Information nonredundant protein database with use of the mascot search program (Matrix Science, London, UK).

Generation and Analyses of Transgenic Mice—DNA fragments containing HSS sequences, VWF-LacZ, and poly(A) were isolated and purified from the plasmids HSS-VWF-LacZ and VWF-LacZ-HSS (described in plasmids generation in supplementary material) by digestion with ASP718 (for plasmid HSS-VWF-LacZ) or Sall (for plasmid VWF-LacZ-HSS). Fragments were used for microinjection to generate C57BL/6 transgenic mice by Ozgene (Bentley, Australia) and University of Alberta transgenic mice facility, respectively. In addition DNA fragment containing VWF-LacZ transgene was used to generate transgenic mice containing VWF-LacZ gene in C57BL/6 mice by Ozgene. All animal housing and experimentation were approved by the Health Sciences Animal Policy and Welfare Committee at the University of Alberta. Two independent lines of HSS-VWF-LacZ and VWF-LacZ, as well as three independent lines of VWF-LacZ-HSS containing the transgenes (determined by PCR) were analyzed. These included one founder line of each HSS-VWF-LacZ and VWF-LacZ-HSS that did not produce progenies and F1 generation of the other lines. Organs, (lung, liver, kidney, brain, heart, stomach, duodenum, cecum, ear, and colon) of founders and F1 lines were harvested and fixed in 4% formaldehyde solution overnight followed by dehydration through graded alcohol, cleared, and tissue-banked as described (18). Tissue macroarrays were prepared by Histobest inc. (Edmonton, Canada). Sectioning was performed at 5 microns, and arrays were subjected to immunohistochemistry using primary anti-LacZ antibody (Abcam ab116-100) at a dilution of 1:500, and secondary anti-mouse IgG antibody (Dako Cytomation Carpinteria, CA) at a dilution of 1:2000 dilution. Normal Rabbit IgG antibody was used as negative control. The reaction products were detected with Envision+/horseradish peroxidase (Dako Cytomation, Carpinteria, CA). Immunostained sections were counterstained with methyl green. In the mouse from one founder line of VWF-LacZ-HSS, the lungs were inflated with 2% paraformaldehyde and cryoprotected with 30% sucrose in phosphate-buffered saline (PBS). Subsequently, 5-micron cryosections were cut, permeabilized with Triton X-100 (0.2% v/v in PBS) for 10 min at room temperature, and washed three times with PBS. Tissue was incubated with rabbit anti-\( \beta \)-galactosidase (U. S. Biological) and rat anti-mouse cd31 (PECAM; clone 390; Pharmingen, San Diego, CA) antibodies for 1 h at room temperature, washed three times in PBS, and labeled with goat anti-rabbit Alexa 488 (Molecular Probes) along with goat anti-rat cy3 (Jackson Immunoresearch Laboratories, West Grove, PA). The slides were mounted in Gelvatol (Monsanto) and visualized using an Olympus 500 confocal microscope. The images were collected with a \( \times 60 \) oil immersion objective at a 1,024 \( \times 1,024 \) pixel resolution. The images were superimposed on differential interference contrast image for aid in structural resolution.

\( \beta \)-Galactosidase and Luciferase Detection in Transfected Cells—Quantitative measurements of \( \beta \)-galactosidase and luciferase expression in transfected cells were performed using the \( \beta \)-Galactosidase Enzyme Assay System and Luciferase Assay System with Reporter Lysis Buffer (Promega) according to the manufacturer’s directions. Immunofluorescence analysis was used to qualitatively detect \( \beta \)-galactosidase expression in transfected freshly isolated mouse pulmonary microvascular endothelial cells. For this analysis mouse pulmonary microvascular endothelial cells grown in Lab-Tek II chambered slides (Nunc) were fixed in 4% paraformaldehyde for 20 min and permeabilized with 0.1% Triton X-100. The cells were stained with an Alexa 555-labeled rabbit anti-mouse polyclonal antibody to \( \beta \)-galactosidase (U. S. Biologicals) and Alexa 647-labeled IB4-isolecistin (Molecular Probes). The images were collected using an Olympus Fluoview 1000 confocal microscope.

RESULTS

Identification of a DNase I-hypersensitive Site within Intron 51 of the VWF Gene—Analysis of the VWF gene promoter and its 5’ proximal region in vivo demonstrated that additional regulatory DNA sequences are required for transcriptional activation of the VWF gene in endothelial cells of all organs. Although cis-acting regulatory elements may be present anywhere within the VWF gene, we chose to investigate whether the 3’ region of the VWF gene contained potential regulatory sequences. The rationale for choosing the 3’ region for this analysis was based on previous reports demonstrating that next to 5’ regions, regulatory elements are most commonly identified at the 3’ end of genes (19–21).

We employed the technique of DNase I-hypersensitive site identification commonly used to locate potential DNA regulatory sequences within large genomic regions. Nuclei were prepared from HUVECs that express VWF and two nonendothelial cell types, HeLa and HUMFIB, that do not express VWF.
isolated nuclei were either untreated or subjected to increasing concentrations of DNase I prior to digestion with EcoRI restriction enzyme and DNA isolation. DNA was then subjected to Southern blot analysis using a radioactively labeled 233-bp DNA probe corresponding to nucleotides 38/3 to 38/235 of the VWF gene (2), including regions of intron 50, exon, and intron 51 (Fig. IA). Based on the previously reported sequence of the VWF gene (2), we expected to detect a 2.4-kb EcoRI DNA fragment following hybridization of the sample that was not treated with DNase I. In both the HUVEC and HUMFIB samples, a 2.4-kb fragment was observed (Fig. 1B, 2.4 kb PB). However, in HUMFIB DNA, an additional fragment of ~300 bp was also detected (Fig. 1B, 0.3 kb DNB). The 300-bp fragment was observed even without addition of DNase I, suggesting that this site is sensitive to endogenous nucleases. Similar results were obtained when HeLa chromatin was analyzed (data not shown).

For HUVEC, although increasing the concentration of DNase I treatment resulted in a decrease in intensity of the 2.4-kb fragment, the level of decrease was significantly lower than that for HUMFIB nuclear DNA, and the 300-bp fragment was not detected (Fig. 1B, lanes 5–9). These results demonstrated the presence of a strong, DNase I-hypersensitive site within intron 51 of the VWF gene in two human, nonendothelial cell types (HeLa and fibroblasts) that was absent in HUVEC.

Identification of Specific Protein-DNA Binding Sequences within the 3′ Fragment—Based on detection of the VWF intron 51-hypersensitive site in nonendothelial cells and its absence in endothelial cells, we hypothesized that the DNase I hypersensitivity may have arisen as a result of DNA-nuclear protein interactions that may function as repressors in nonendothelial cells. To test this hypothesis, we first determined whether specific protein-DNA complex formation occurs at the intron 51-hypersensitive site (I51-HSS) in nonendothelial cells. We carried out electrophoretic mobility shift assays (EMSA) utilizing nuclear extracts from human fibroblast cells and a 205-bp probe encompassing the DNase I-hypersensitive site. The probe corresponded to sequences 38/231 to 38/435 of the VWF gene (2). Several protein-DNA complexes (Fig. 2, C1–C3) formed with this fragment that were abolished or significantly reduced in the presence of specific competitor, whereas a nonspecific DNA competitor did not affect these complex formations (Fig. 2, lanes 1–3). To localize the binding sequences of the nuclear protein(s) in this 205-bp DNA sequence, smaller DNA fragments corresponding to various regions within this DNA fragment were used as competitors in EMSA. Specific protein-DNA interactions sites (specifically complexes C2 and C3) were localized to nucleotides 38/280 to 38/380 (Fig. 2, lanes 2–11). The specific competitor is the unlabeled DNA fragment 231–435, and the nonspecific (NS) competitor is an unrelated 202-bp DNA fragment. DNA fragments ranging in size from 30 to 205 base pairs corresponding to various regions of the fragment 231–435 were used as competitors.
observed in repeated assays. Thus based on consistency and the prominence of the specific protein-DNA interactions that occurs with nucleotides 380–409, we first chose to pursue characterization of this protein complex.

Protein Complexes That Include YY1 Transcription Factor Interact with DNA Sequences in the Intron 51 DNase I-hypersensitive Region—Based on observations that I51-HSS was specifically detected in nonendothelial cells and the hypothesis that the proteins interacting with I51-HSS sequences may function as a repressor, we proceeded to determine whether there are differences in the presence/absence or the pattern of the observed specific protein-DNA complexes that form with nucleotides 380–409 in nonendothelial compared with endothelial cells. We carried out gel mobility experiments using a double-stranded oligonucleotide corresponding to nucleotides 38/380–38/409 as a probe and nuclear extracts from HUVECs and HUMFIB and HeLa cells. The results demonstrated that nuclear proteins from both cell types form a complex that migrates to a similar position in an acrylamide gel (referred to as CC). In addition to this, cell type-specific complexes with DNA sequences in the hypersensitive region of the VWF gene intron 51 were formed with HUVEC and HUMFIB nuclear extracts, respectively. CC indicates the specific DNA-protein complex that was similarly formed with nuclear extracts of all cell types. B, competition EMSA analysis using competitor oligonucleotides containing different four nucleotide base substitutions in the 380–409 fragment as shown in C. The labeled wild type probe (380–409 oligonucleotide) was incubated with HUVEC or HUMFIB nuclear extracts in the absence (lane 1) or presence of 100-fold excess oligonucleotide competitors. The unlabeled 380–409 oligonucleotide was used as a specific competitor (S), and an unrelated 30-bp oligonucleotide was used as a nonspecific competitor (NS), HVC and HFC indicate the DNA-protein complexes that were uniquely formed with HUVEC, and HUMFIB or HeLa nuclear extracts, respectively. CC indicates the specific DNA-protein complex that was similarly formed with nuclear extracts of all cell types. B, competition EMSA analysis using competitor oligonucleotides containing different four nucleotide base substitutions in the 380–409 fragment as shown in C. The labeled wild type probe (380–409 oligonucleotide) was incubated with HUVEC or HUMFIB nuclear extracts in the absence (lane 1) or presence of 100-fold excess mutant 30-bp double-stranded oligonucleotides m1, m2, m3, or m4. C, schematic representation of the nucleotide sequences of the wild type and mutant 380–409 (m1-m4) double-stranded oligonucleotides with the base substitutions shown.

To identify the nucleotides within the 380–409 DNA fragment necessary for the formation of various complexes, we generated additional sets of oligonucleotides containing different four nucleotide substitutions (Fig. 3C). The only mutant oligonucleotide that did not significantly compete with the wild type probe and hence was unable to abolish complex formations was the m2 oligonucleotide (Fig. 3B). Based on these results, a core DNA sequence, AATG, was identified as necessary for formation of specific protein-DNA complexes. These results also demonstrated that the same core sequence was necessary for the formation of all three (CC, HVC, and HFC) complexes.

Using a data base containing transcription factor binding consensus sites, we determined that the sequence AATGG and the surrounding nucleotides (AA AATG G) were homologous to the core consensus binding site of the transcription factor YY1 (Yin Yang 1). To determine whether YY1 was interacting with this core sequence, we carried out competition and supershift experiments using sequences 380–409 as probe, oligonucleotides containing the consensus YY1 binding sequence (corresponding to that of murine immunoglobulin heavy-chain intronic enhancer (μE1)) as competitor, and anti-YY1 antibody. The results demonstrated that all three complexes were abolished in the presence of the YY1-specific competitor (Fig. 4A, lanes 3 and 5) and were supershifted in presence of the anti-YY1 antibody (Fig. 4B). These data demonstrate that YY1 interacts with the intron 51 sequence AATGG, and both the common and cell specific complexes formed with HUVEC and human fibroblast nuclear extracts contain YY1.

Intron 51 HSS Sequences Target the Activation of the VWF Promoter in a Subset of Lung Endothelial Cells in Vivo—VWF promoter sequences −487 to +247 activate transcription only in endothelial cells of the brain but not other organs. To determine whether the 380-bp DNA region (referred to as I51-HSS) that encompasses the DNase I-hypersensitive and YY1-binding site participates in regulation of VWF transcription in vivo, we generated transgenic mice harboring a LacZ transgene driven by the VWF promoter (sequences −487 to +247) and the I51-

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FIGURE 3. Nuclear proteins from HUMFIB cells and HUVEC form common and cell type-specific complexes with DNA sequences in the hypersensitive region of the VWF gene intron 51. A, nuclear extracts (10 μg) from HUVEC and HUMFIB and HeLa cells were incubated with the labeled 380–409 oligonucleotide as probe (10,000 cpm) in the absence (lane 1) or presence of 100-fold excess oligonucleotide competitors. The unlabeled 380–409 oligonucleotide was used as a specific competitor (S), and an unrelated 30-bp oligonucleotide was used as a nonspecific competitor (NS). HVC and HFC indicate the DNA-protein complexes that were uniquely formed with HUVEC, and HUMFIB or HeLa nuclear extracts, respectively. CC indicates the specific DNA-protein complex that was similarly formed with nuclear extracts of all cell types. B, competition EMSA analysis using competitor oligonucleotides containing different four nucleotide base substitutions in the 380–409 fragment as shown in C. The labeled wild type probe (380–409 oligonucleotide) was incubated with HUVEC or HUMFIB nuclear extracts in the absence (lane 1) or presence of 100-fold excess mutant 30-bp double-stranded oligonucleotides m1, m2, m3, or m4. C, schematic representation of the nucleotide sequences of the wild type and mutant 380–409 (m1-m4) double-stranded oligonucleotides with the base substitutions shown.

FIGURE 4. YY1 interacts with DNA sequences in the hypersensitive region of the VWF gene intron 51. A, nuclear extracts from HUVEC and HUMFIB (5 μg) cells were incubated with labeled 380–409 oligonucleotide probe in the absence (−) or presence of an oligonucleotide competitor containing the consensus YY1-binding site (YY1). B, supershift gel mobility experiments were carried out using HUVEC or HUMFIB nuclear extracts (5 μg) and the wild type 380–409 oligonucleotide probe in the absence (−) or presence of nonspecific IgG (NS) or anti-YY1 (YY1) antibodies (0.5 μg).
The results demonstrated that LacZ transgene was expressed in the brain vasculature in transgenic mice. The expression was restricted to endothelial cells of these organs, because the Wt promoter sequence was previously shown to target activation in brain vascular endothelial cells. In the lung vasculature, immunofluorescent analysis performed using specific antibodies to detect LacZ gene products in various organs. We analyzed one founder line of the Wt mouse, respectively, using confocal microscopy. Overlapping windows (yellow) demonstrated colocalization of both PECAM and β-galactosidase in brain and lung endothelial cells (Fig. 6C). Similarly, LacZ expression was not detected in the kidney (ependymal cells) of one line of Wt-LacZ and HSS-LacZ. These were attributed to escape expression, possibly by 30% survival (one founder line for Wt-LacZ-HSS). Tissue arrays of various organs were prepared on a single slide for analysis. Some β-galactosidase staining was observed in the colon (mononuclear cells) of one line of HSS-LacZ-WT. These were attributed to escape expression, possibly by 30% survival (one founder line for Wt-LacZ-HSS). Tissue arrays of various organs were prepared on a single slide for analysis.
endothelial cells, these results demonstrate that the lung-endothelial specific activation of the chimeric promoter, containing I51-HSS sequences and sequences 487 to +247, is due to the regulatory function of the I51-HSS sequences.

**The Effect of the Intronic 51 HSS Sequences on Gene Expression Driven from Homologous and Heterologous Promoters in Endothelial and Nonendothelial Cells in Vitro**—To determine whether I51-HSS sequences have a differential and potentially cell type-specific function in regulating VWF promoter in endothelial and nonendothelial cell types, we determined the transcriptional activity of the homologous VWF promoter (sequences 487 to +247) and a heterologous SV40 early promoter in the absence and presence of I51-HSS sequences, in HUVEC and sheep pulmonary endothelial cells as well as HeLa cells. Because VWF promoter fragment (487 to +247) is not activated in nonendothelial cells, to test the hypothesis that I51-HSS may function as a repressor in non-endothelial cells, we chose to determine its effect on the activity of a heterologous SV40 promoter. Plasmids containing human growth hormone or luciferase reporter genes, as well as the plasmids expressing LacZ gene (VWF-LacZ, HSS-VWF-LacZ, and VWF-LacZ-HSS described above) were used for these analyses. I51-HSS were positioned upstream of the VWF promoter in plasmid HGH-K (the resulting plasmid was designated 3'-HGHK) or upstream of the SV40 promoter fused to luciferase gene in the plasmid pGL3pr (to generate the plasmid 3'-pGL3pr) (Fig. 7A).

We compared the expression pattern of HGH-K to 3'-HGHK in transiently transfected HUVECs. HUVECs are unable to undergo the extended cell divisions necessary for producing stable transfectants; thus we used a transient transfection assay for this cell type. The reporter growth hormone (from HGH-K and 3'-HGHK) expressions were determined 48 h post-transfection. To control for transfection efficiency, a β-galactosidase reporter plasmid was cotransfected with all test plasmids. The results demonstrated that addition of the YY1 containing I51-HSS sequences did not significantly affect the homologous VWF promoter activity in HUVECs (Fig. 7B). However, *in vivo* analyses demonstrated a lung-specific activity for I51-HSS sequences; thus we explored the effect of I51-HSS sequences on the VWF promoter activity in cultured lung endothelial cells *in vitro*. Sheep pulmonary artery endothelial cells were transiently transfected with the plasmids VWF-LacZ, HSS-VWF-LacZ, and VWF-LacZ-HSS. To control for transfection efficiency, a luciferase reporter plasmid was cotransfected with all test plasmids. β-Galactosidase activity (using enzymatic detection assay) in transfected cells extracts were determined by enzymatic detection as well as immunofluorescent staining 48 h post-transfection. Enzymatic detections were used for quantitative assessment of the levels of LacZ expression and were normalized to the levels of cotransfected luciferase.

The results of quantitative assessments demonstrated that there was no significant quantitative effect of HSS sequences on the VWF promoter activity in these pulmonary endothelial cells in culture (Fig. 7C). Similar results were obtained when the
effect of HSS on VWF promoter activity was tested in human pulmonary arterial endothelial cells and bovine pulmonary endothelial cells (data not shown). Immunofluorescent staining with anti-β galactosidase antibody demonstrated that VWF-LacZ, HSS-VWF-LacZ, and VWF-LacZ-HSS were all expressed in pulmonary endothelial cells in culture (Fig. 7D). These results demonstrate that although VWF promoter sequences −487 to +247 are not active in lung endothelial cells in vivo, these sequences exhibit promoter activity in lung endothelial cells as well as other endothelial cell types in vitro and that addition of I−51 sequences does not enhance the level of VWF promoter activity in endothelial cells in culture.

Next we determined the effect of I51-HSS sequences on the heterologous SV40 promoter activity in HUVECs and HeLa cells. Plasmids pGL3pr, 3′-pGL3pr, and 3′-M-pGL3pr were transiently transfected in HUVECs and stably transfected into HeLa cells. Heterogeneous population of stably transfected HeLa cells were analyzed for luciferase expression, whereas luciferase expressions were determined 48 h post-transfection in HUVECs. To control for transfection efficiency, a β-galactosidase reporter plasmid was cotransfected with all test plasmids. The results demonstrated that the activity of the minimal SV40 promoter was significantly increased by the presence of I51-HSS sequences in HUVECs, whereas it was significantly decreased in HeLa cells (Fig. 7, E and F). The enhancing effect of I51-HSS sequences on the SV40 promoter in HUVECs was dependent on an intact YY1-binding site, because mutation of the YY1-binding site in the plasmid 3′-M-pGL3pr (Fig. 7A) significantly reduced luciferase gene expression compared with the wild type 3′-pGL3pr (Fig. 7E). However, mutation of YY1-binding site did not abrogate the inhibitory function of I51-HSS (Fig. 7F).

These analyses demonstrated that the I51-HSS sequences did not significantly affect the homologous VWF promoter activity, whereas they were capable of conferring endothelial specific activation and nonendothelial cell-specific repression on the heterologous SV40 promoter. These results also demonstrate that interaction of YY1 with its cognate binding site in I51-HSS was required (although it may not be sufficient) for the activating function of this fragment in endothelial cells. However, the repressive function of I51-HSS in nonendothelial cells is either independent of YY1, or additional repressors may be present in this region, which could also contribute to its repressive function.

**YY1 Interacts with Actin in a Cell Type-specific Manner**—Based on YY1-dependent activating effect of I51-HSS sequences on SV40 promoter in HUVECs and the repressive effect of I51-HSS in HeLa cells as well as differential YY1-DNA complex formation in the two cell types, we hypothesized that YY1 may differentially interact with nuclear proteins in endothelial and nonendothelial cells. To test this hypothesis, we immunoprecipitated YY1 from nuclear extracts of HUVECs, HeLa, and another nonendothelial cell type, namely HEK 293 cells. The immunoprecipitates were analyzed on SDS-PAGE gels to determine whether additional proteins that interact with YY1 are detected as coimmunoprecipitates. The results demonstrated that a prominent polypeptide at ~42 kDa (shown by arrow) was detected in YY1 immunoprecipitates of HeLa and HEK293 cells that was not detected in HUVECs (Fig. 8A). The region containing this polypeptide was cut from the gel and subjected to mass spectrometry. The results demonstrated that this polypeptide has sequence homology to β-actin (Fig. 8B).

To confirm whether β-actin is associated with YY1, we performed Immunoprecipitation/Western blot analysis. YY1 was immunoprecipitated from nuclear extracts prepared from HUVEC, HeLa and HEK 293 cells and subjected to SDS-PAGE analysis followed by Western blot analysis with anti-β-actin antibody and then as a control for immunoprecipitation with anti-YY1 antibody. The results demonstrated that YY1 that was immunoprecipitated from all three cell types associates with β-actin in HeLa and HEK293 but not in HUVECs cells (Fig. 8C). Western blot analysis to detect β-actin in nonimmunoprecipitated nuclear extracts of HUVEC, HeLa, and HEK 293 cells demonstrated comparable levels of nuclear β-actin in all three cell types (data not shown), thus confirming that the lack of β-actin association with YY1 in HUVECs was not due to the absence of either YY1 or β-actin in nuclear extracts of HUVECs. To determine whether DNA-YY1 complexes that are observed in gel mobility assays also contain β-actin, we next performed supershift gel mobility assays (as described for Fig. 4B) using sequences 380–409 as probe with nuclear extracts prepared from HUVEC and HeLa cells and anti-β-actin antibody. The results demonstrated the presence of a supershifted complex with anti-β-actin antibody when nuclear extracts from HeLa cells were used but not with HUVECs nuclear extracts (Fig. 8D). These data demonstrate that β-actin is a component of the YY1-containing complexes that interact with the intron 51 sequences in HeLa cells but not HUVECs.

**DISCUSSION**

Under in vivo conditions, endothelial cells display marked heterogeneity in structure and function. Local microenvironment is a major participating factor in establishment of endothelial cell heterogeneity, to an extent that when they are removed from their native environment and grown in culture, endothelial cells undergo phenotypic drift and lose many of their organ-specific characteristics (22–24). Analyses of the activation pattern of endothelial specific promoters in vitro and in vivo also reflect the heterogeneity of endothelial cells. Although endothelial specific promoters in vitro demonstrate cell type specificity of function, in many cases in vivo analyses demonstrate that analyzed promoter regions of endothelial specific genes carry information for expression in distinct subsets of endothelial cells or vascular beds (25–28).

Results of the analyses of the VWF promoter activity in transgenic mice to date are specifically reflective of the endothelial subset-specific function of distinct regions of the VWF gene. Analyses of two overlapping regions of the VWF promoter lead to identification of a brain-specific region and a distinct region that is necessary for heart and muscle activation (11, 12). Because VWF gene is expressed in endothelial cells of many organs, this suggested that additional VWF gene sequences might be necessary for activation of transcription in other organs. Previous reports have demonstrated that DNA regulatory elements are located in introns and 3′ regions of some genes, as well as the 5′ proximal and distal sequences to the
transcription initiation site. Endothelial specific enhancers for several genes including Tie-2, flk1, and recently GATA2 were identified downstream of transcription initiation site, ranging in location from 3’ region of intron 1 to intron 4 (29–31). Thus we chose to explore the 3’ region of the VWF gene for the presence of potential regulatory elements. Using DNase 1-hypersensitive assay as an initial screening technique, we identified a region in intron 51 of the VWF gene that was hypersensitive to DNase I digestion in nonendothelial human fibroblast and HeLa cells but not in HUVEC. We demonstrated that this region (I51-HSS) interacts with distinct endothelial specific and nonendothelial specific YY1-containing protein complexes.

We determined the effect of I51-HSS on the VWF promoter activity in transgenic mice. Because generation of a transgene that mimicked endogenous VWF with regard to the position of I51-HSS relative to the promoter was not feasible, we generated transgenic mice containing the I51-HSS placed either upstream or downstream of the VWF promoter sequences −487 to +247

![FIGURE 8. β-Actin is associated with YY1 specifically in nonendothelial cells.](image)

A, nuclear proteins (300 μg) from HUVEC, HEK 293, and HeLa cells (lanes 5–7) were immunoprecipitated with anti-YY1 antibody (Ab) or rabbit IgG (lane 4), and the immunoprecipitates were analyzed on SDS-PAGE by Coomassie Blue staining. Lane 1 represents the protein molecular weight marker, and lanes 2 and 3 represent proteins from HEK 293 and HUVEC nuclear extracts (100 μg) that were not immunoprecipitated. The position of immunoprecipitated YY1 is shown by an asterisk, and the position of the 42-kDa coimmunoprecipitated polypeptide is shown by an arrow. B, mass spectrometry analysis of the 42-kDa polypeptide (shown by an arrow in A) from both HEK 293 and HeLa cells were carried out. Amino acid sequence of β-actin is shown. The sequences in red represent the peptides identified by mass spectrometry analysis of the 42-kDa polypeptide. C, immunoprecipitation (IP) of nuclear extracts (150 μg) represented as nuclear extract (N.E.) from the three cell types were carried out as described in A and then subjected to Western blot analysis with anti-β-actin antibody (W: actin) and subsequently with anti-YY1 antibody (W: YY1). The IgG antibody used as nonspecific antibody was mouse IgG. Lane 1 represents 20 μg of total nuclear extracts from HEK cells used as input control, which demonstrates the presence of β-actin and YY1 in HEK cells. D, supershift gel mobility experiments were carried out using HUVEC or HeLa nuclear extracts (10 μg) and the wild type 380–409 oligonucleotide probe in the absence (−) or presence of nonspecific IgG (NS), anti-YY1 (YY), or anti-β-actin antibodies (1 μg). The positions of YY1 containing complexes (HFC and CC) are shown by arrows. The lack of HVC detection in this experiment may be due to lower specific activity of the probe used, which could hamper detection of minor complexes (relative intensity of HVC band is significantly lower than that of HFC and CC as shown in Figs. 3 and 4. The positions of YY1 supershifted (YY SS) and β-actin supershifted (actin SS) complexes are shown by arrows.
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(HSS-VWF-LacZ and VWF-LacZ-HSS) that regulated the expression of the LacZ transgene. Generation of the transgene containing I51-HSS positioned immediately upstream of the VWF promoter (HSS-VWF-LacZ) was based on the hypothesis that these sequences may naturally exert their effect on the promoter via chromatin looping. Chromatin looping is reported to be one mechanism that could bring elements located at a long distance from transcription initiation site of a gene to close proximity of its proximal promoter and thus affect transcription (32). However, I51-HSS is located at the 3’ region of the VWF gene and its location with regard to being positioned downstream versus upstream of transcription initiation site may influence its function, we also generated the transgene that contained I51-HSS positioned downstream of the VWF promoter (VWF-LacZ, HSS).

Analysis of the transgenic mice demonstrated that independent of positioning, I51-HSS sequences activated the VWF promoter in a subset of lung endothelial cells as well as brain microvascular endothelial cells (as was expected from the activity of the VWF promoter sequences −487 to +247). The expression pattern of the LacZ in lungs of these transgenic mice was highly heterogeneous and in large part was restricted to a subset of endothelial cells in the parenchyma near the pleura. This most likely reflects the requirement for additional VWF gene sequences to mimic endogenous VWF expression pattern. It may also partly reflect the heterogeneous nature of endogenous VWF gene expression in lung. Reports of endogenous VWF gene expression in human and mouse have demonstrated that VWF expression in lung is not uniformly observed in all endothelial cells (33–37). Notably, the VWF promoter sequences −487 to +247 also do not uniformly activate the LacZ transgene expression in all brain vascular endothelial cells, thus suggesting that distinct regions of the VWF gene identified to date may contain the minimal necessary elements for transcriptional activation in vasculature of specific organs, but additional sequences may also contribute to enhancement/activation in a broader range of endothelial cells in these organs.

The narrow range of activation by I51-HSS that is specific to the lung suggests that elements that are necessary for VWF promoter activation in vasculature of all organs are not clustered together and may be scattered throughout the VWF gene. This hypothesis is consistent with the observation that addition of the sequences −2182 to −487 and intron 1 to the brain-specific VWF promoter sequences results in promoter activation only in two additional organs, heart and muscle. Alternatively, the narrow specificity may reflect detection of partial function of I51-HSS when studied out of their native context. The I51-HSS sequences in their native position in relation to the promoter could have additional functions that may not have been detected by these analyses.

Analysis of the function of I51-HSS sequences in vitro demonstrated that these sequences confer endothelial specific activation to a heterologous SV40 promoter, although it represses its activity in nonendothelial cells. However, I51-HSS had no significant effect on the activity of the homologous VWF promoter in either HUVECs or lung endothelial cells in culture. These results suggest that organ-specific characteristics of the VWF promoter may not be detected in vitro. The in vivo lack of promoter activity (sequences −487 to +247) in lung endothelial cells may be the prerequisite for recognizing a role for I51-HSS sequences in the VWF promoter activation in lung endothelial cells. We propose that I51-HSS sequences function as a lung endothelial specific activator of transcription. This specific function of I51-HSS sequences could not be detected in vitro using the homologous VWF promoter, because the promoter is already active in endothelial cells in culture regardless of organ-origin. We hypothesize that the observed enhancing effect of the I51-HSS sequences on heterologous SV40 promoter is due to a minimal level of SV40 promoter activity in HUVECs, which may allow the manifestation of an endothelial specific activating function of I51-HSS. That we observed this effect in HUVECs, whereas the activity of I51-HSS in vivo is restricted to lung endothelial cells, may reflect the loss of organ specific activity of the HUVECs in culture, especially with regard to regulation of VWF transcription. This is supported by observation that the −487 to +247 VWF promoter, which is only active in brain endothelial cells in vivo, is also active in HUVECs in vitro. Hence if I51-HSS mainly functions as an endothelial specific activator but not necessarily an enhancer, it may not further increase VWF promoter activity where it is already active.

Notwithstanding the caveat of being a heterologous promoter, analyses of I51-HSS sequences in relation to the basal SV40 promoter in vitro provide some clues toward identifying regulatory elements in these sequences that may contribute toward its function as an endothelial specific activator. Mutation analysis demonstrated that endothelial specific activating function of I51-HSS (in regard to SV40 promoter) is dependent on an intact YY1-binding site, whereas its repressive function in nonendothelial cells is not abolished as a result of the mutation in YY1-binding site. This suggests that although YY1 function as an endothelial specific activator of I51-HSS, either the repressive function of this region in nonendothelial cells is completely independent of YY1, or additional repressor elements are also present that participate in inhibitory function of this region. We have demonstrated that the VWF promoter sequences −487 to +247 contain multiple repressors that inhibit its activation in nonendothelial cells, and inhibition of a single repres sor binding site is not sufficient to relieve repression (7). A similar presence of multiple repressor sites, one of which could be YY1, may also be the mechanism that regulates the repressive function of HSS intron 51 sequences in nonendothelial cells. Previous reports of YY1 as a transacting factor with dual activating and repressive function is consistent with a potential repressor and activator function for YY1 in regulating I51-HSS function (38, 39). We hypothesize that the endothelial specific YY1-containing complex (HVC) may function as an activator, whereas the nonendothelial specific YY1-containing complex (HFC) may function as a repressor of I51-HSS activity or that such a complex, even if it does not directly function as a repressor, may inhibit the activating function of YY1.

Analysis of proteins that communoprecipitate with YY1 in HUVECs, HeLa, and HEK 293 cells demonstrated that β-actin is associated with YY1 specifically in the nucleus of nonendothelial cells and is a constituent of the YY1-containing complexes in these cells that interact with I51-HSS, whereas this
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association was not observed in HUVECs. Recently a role for actin monomer in the nucleus as a regulator of transcription has been reported (40, 41). Vartiainen et al. (40) demonstrated that nuclear actin monomer (specifically β-actin) interacts with MAL (a myocardin family transcriptional coactivator for serum response factor) and functions to repress its activity. Our data demonstrating that actin is associated with YY1 specifically in nonendothelial cells is consistent with a hypothesis that association of β-actin with YY1 may confer a repressor function to YY1 when interacting with I51-HSS. In endothelial cells the lack of this association (and potentially association with another cofactor) may render YY1 as an activator when interacting with I51-HSS. Observed selective hypersensitivity of I51-HSS region in nonendothelial cells suggests that the interaction of nonendothelial YY1-containing complex (including β-actin) with intron 51 sequences may induce a chromatin configuration that is specifically hypersensitive to digestion with DNase I.

Despite the lack of uniform vascular expression in lungs, our results demonstrate that regulatory elements in intron 51 of the VWF gene confer lung-specific activation to the VWF promoter in vivo. Because VWF is expressed by nearly all endothelial cells, the findings that endothelial cells in distinct vascular beds rely on different mechanisms for VWF transcriptional regulation may seem odd on the surface, but it is consistent with growing evidence that distinct regions in a number of endothelial cell subtypes (25–28). Site-specific regulation of endothelial VWF transcription could reflect distinct signals acting with I51-HSS. Observed selective hypersensitivity of I51-HSS may render YY1 as an activator when interacting with another cofactor).

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