An NF1-like Protein Functions as a Repressor of the von Willebrand Factor Promoter*

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Nadia J. ahroudi, Ali M. Ardekani, and Joel S. Greenberger

From the Department of Radiation Oncology, University of Pittsburgh Medical Center and University of Pittsburgh Cancer Institute, Pittsburgh, Pennsylvania 15213

The expression of the von Willebrand factor (vWf) gene is restricted to endothelial cells and megakaryocytes. We have previously reported the identification of a region of the vWf gene that regulates its cell-type-specific expression in cell culture. This region (spanning nucleotides −487 to +247) consists of a core promoter (spanning nucleotides −90 to +22), a positive regulatory region (spanning nucleotides +155 to +247), and a negative regulatory region spanning nucleotides −312 to −487. To identify the trans-acting factor(s) that interact with the negative regulatory region, we carried out gel mobility and DNaseI footprint analyses of sequences −312 to −487. These analyses demonstrated that an NF1-like protein interacts with DNA sequences spanning −440 to −470 nucleotides in the negative regulatory region of the vWf promoter. Base substitution mutations of the NF1 binding site abolished the NF1-DNA interaction. Furthermore, mutation of the NF1 binding site in the promoter fragment (−487 to +155) that contained the core and the negative regulatory region resulted in activation of the mutant promoter in both endothelial and nonendothelial cells. The wild type promoter fragment (−487 to +155) was not activated in either cell type. These results demonstrate that an NF1-like protein functions as a repressor of vWf promoter activity. In contrast, the mutation of the same NF1 binding site, but now in the context of the larger 734-base pair endothelial cell-specific promoter fragment (−487 to +247), did not result in promoter activation in nonendothelial cells. The data indicate that there are additional repressor elements within the vWf promoter region suppressing its activity specifically in nonendothelial cells, and suggest that there is a secondary repressor element(s) that is located in the terminal region of the first exon of this gene.

Endothelial cells are known to have a fundamental role in a variety of physiological and pathophysiological processes including tissue homeostasis, blood tissue exchange, thrombosis, angiogenesis, and cancer metastasis (1–6). Therefore, defining the basic molecular determinants of the endothelial cell phenotype is important to a variety of clinical and basic science disciplines (6). The molecular mechanism that controls the lineage specificity of endothelial cells is not well understood. One approach toward understanding the lineage specificity of endothelial cells is through investigation of the mechanism that regulates transcription of endothelial cell-specific genes, such as the gene that codes for von Willebrand factor (vWF).

The protein vWF is a glycosylated protein that circulates in blood plasma as multimers of up to 100 subunits (7). The vWF gene is located on chromosome 12, spans −178-kilobase pairs, and is interrupted by 51 introns (8). The biosynthesis and secretion of vWF have been extensively studied (7, 9, 10). Expression of the vWF is highly restricted to endothelial cells and megakaryocytes and is most often used as a marker for endothelial cell-specific differentiation (11). In addition, vWF has a central role in at least two major aspects of hemostasis, mediation of platelet interaction with damaged endothelial surfaces and stabilization of factor VIII in the circulation (7, 12). Investigation of the mechanism of endothelial cell-specific regulation of the vWF gene may also provide insight into the nature of the molecular defects that result in vWF quantitative deficiencies in patients with von Willebrand disease.

The lineage specificity of differentiated cells has been proposed to depend upon the cell-type-specific distribution of various transcription factors (13). Through interactions with cis-acting DNA elements located in the promoter or enhancer regions of a cell-specific gene, such transcription factors regulate the expression of genes that are specific to that particular lineage. This molecular regulatory system is best exemplified by GATA transcription factors in erythroid specific hematopoiesis (14). Until recently most emphasis has been on the factors that positively regulate gene expression. There is increasing evidence to suggest that trans-acting factors that negatively regulate gene expression are also important in determining cell lineage specificity (15, 16). The presence of negative regulatory elements and repressors has been reported in many genes including those for yeast mating type (17), chicken lysozyme (18), rat growth hormone (19), and others (15, 16). In addition it has been shown that trans-acting factors such as hormone receptors can function both as repressors and activators depending on the sequence context of the promoters (16, 20). Direct evidence for the role of repressors in regulating cell-type-specific gene expression was provided by the recent report of Chong et al. (21) that describes a repressor protein REST. The REST protein is expressed in all cell types except those of neuronal origin and inhibits the expression of the sodium channel gene in all cells, except those of neuronal origin (21).

One direct method to identify both positive and negative factors that may regulate the endothelial cell-type-specific expression of the vWF gene would be to identify the cis-acting elements that regulate gene expression. Such cis-acting regulatory elements may then be used to identify and characterize the trans-acting factors that bind to them. We have identified a
region of the vWF gene spanning sequences –487 to +247 bp that mediates the endothelial cell-specific expression of a heterologous reporter gene in cell culture (22). This region consists of a core promoter spanning –90 to +22 bp (+1 being the transcription start site) which is not cell-type-specific, a negative regulatory region located within –312 to –487 bp, and a positive regulatory region located in the 100-bp terminal sequences of the first exon (+150 to +247). We have shown that the negative regulatory region inhibits transcription in all cell types studied. The positive regulatory region is required to overcome the inhibitory negative regulator in endothelial cells. Furthermore, an intact GATA binding site in the positive regulatory region is necessary for endothelial cell-specific activation of the promoter (22).

We now report that an NF1-like protein (23) interacts with its cognate cis-acting element in the negative regulatory region of the vWF promoter and through this interaction inhibits promoter activity in both endothelial and nonendothelial cells. We also report that there are additional negative regulatory element(s), located in the terminal region of the first exon, that inhibit vWF promoter activity in nonendothelial cells.

MATERIALS AND METHODS

Plasmid Constructions—Generation of the plasmids HGH-1, HGH-B, and HGH-K are previously described (22). To generate plasmids HGH-Brm3 and HGH-Krm3, appropriate fragments of the vWF gene (–487 to +155 for HGH-Brm3 and –487 to +247 for HGH-Krm3) were amplified using PCR with 49- and 39-mer oligonucleotides as primers. These oligonucleotides contained 40 and 30 bases that were complementary to the region of interest, and the remaining 9 bases at the 5′ end of each oligonucleotide were located in the SalI restriction site for cloning. Oligonucleotides complementary to –474 to –477 containing the 3′-bp substitution at positions –452, –453, and –454 were used in combination with oligonucleotide complementary to +125 to +155 to generate Brm3 fragment and +217 to +247 to generate Krm3 fragment. The resulting fragments were cloned into the unique SalI site upstream of the growth hormone structural gene in plasmid pGEMH (obtained from Nichols Institute Diagnostics). The resulting HGH-Brm3 and HGH-Krm3 plasmids were sequenced using the Promega Sequencing kit that confirmed the presence of the required three-base substitutions, with no other mutations in the vWF fragments.

Cell Culture, Transfections, and Growth Hormone Assays—Primary bovine aorta endothelial (BAE) cells and bovine smooth muscle (BSM) cells were maintained as described previously (22). Primary cells, between passages 7 and 12, were used for transfection at 50–70% confluency. Transfections were carried out by the calcium phosphate coprecipitation method (24). The precipitates were left on BAE cells for 6 h and then on BSM and HeLa cells (22). The activity of this nonspecific repressor protein(s) in the negative regulatory region of the vWF promoter was inhibited by sequences spanning 5′-end-labeled probe spanning nucleotide 550 to –300 sequences. The probe was generated using PCR and primers that corresponded to –300 to –330 and –550 to –520. The primer corresponding to –300 to –330 was radioactively labeled using polynucleotide kinase and γ-32P. The 5′-end-labeled probe (10,000 cpm) and negative regulatory region were incubated on ice for 30 min in the presence of 2.5 mM CaCl2, 5 mM MgCl2, and 2 μg of poly(dI-dC). Following incubation samples were treated with 10, 20, and 50 μg/ml Dnase1 for 5 min at room temperature. The Dnase1 digestion was terminated by addition of 2 μl of 0.5 M EDTA, and DNA was purified by phenol/chloroform extraction and ethanol precipitation. Purified DNA was analyzed on 8% polyacrylamide sequencing gel. A sequencing reaction of the same labeled DNA fragment was run in parallel on the same gel. For gel mobility experiments the double-stranded oligonucleotides used as probes corresponded to –470 to –500, –410 to –440, and wild type or mutated (3-bp substitution) –440 to –470 sequences. Oligonucleotides corresponding to wild type and mutated NF1 binding site of the adenovirus replication of origin were as described (23, 29). The sequence of the wild type and mutant adenovirus NF1-containing oligonucleotides was as shown. The consensus recognition sequence is underlined. The bolded sequence in the mutant oligonucleotide represents the base substitutions. A, wild type, CCTTAATTGCAGAAGCCATTGATAAG; mA mutant, CCTTATTATCCAGGAGCATTAATGAAAT.

Binding reactions were carried out in the presence (100 ×) or absence of competitors (wild type or mutant oligonucleotides). The 5′-end-labeled probes (5000 cpm, approximately 3 × 106 cpm/mg) were incubated with 3 μg of nuclear extract, 1 μg of poly(dI-dC), 0.5 mM dithiothreitol in a buffer containing (final concentration) 10 mM HEPES (pH 7.8), 50 mM KCl, 5 mM MgCl2, 1 mM EDTA, and 5% glycerol. The reaction mixture, in a final volume of 20 μl, was incubated on ice for 20 min, and DNA-protein complex was separated from unbound probe on a 5% polyacrylamide gel run in 0.5 × TBE (90 mM Tris borate (pH 8.2), 2.5 mM EDTA) at 4°C.

RESULTS

Identification of DNA Sequences That Interact with Protein(s) in the Negative Regulatory Region of the vWF Promoter—We have previously reported that the region of the vWF gene spanning nucleotides 90 to +155 can function as a promoter and drive the expression of a heterologous growth hormone gene in bovine endothelial cells, bovine smooth muscle cells, and HeLa cells (22). The activity of this nonspecific promoter was inhibited by sequences spanning 312 to –487 nucleotides in all three cell types studied (22). We hypothesized that this inhibition was accomplished through binding of a repressor protein(s) that recognizes a specific cis-acting DNA element(s) located somewhere within this approximately 200-bp region.

To determine whether there is a protein that interacts with DNA sequences in the negative regulatory region, a footprint analysis was carried out. The DNA probe for these analyses was a 5′-end-labeled fragment corresponding to –320 to –550 sequences. The PCR-generated radioactive-labeled fragment was incubated with 30 μg of nuclear extract prepared from BAE cells as described under “Materials and Methods.” The DNA/protein mixtures were digested with various concentrations of Dnase1, and purified DNA was analyzed on an 8% polyacrylamide sequencing gel. The results of these analyses indicated the presence of a protected region covering –442 to –471 sequences (Fig. 1). A protected region that covered sequences –500 to –530 was also observed; however, this was not further analyzed since this region was not included in the promoter fragments that we have studied. These data establish that the region spanning sequences –440 to –470 of the vWF promoter interacted with a protein(s).
incubated with nuclear extract (30 μg), corresponding to sequences spanning nucleotides −320 to −350, and an unlabeled primer that corresponded to the sequence −500 to −550. DNA probe (10,000 cpm) was incubated with nuclear extract (30 μg) from BAE cells for 15 min on ice and digested with DNase I enzyme: 0, lane 1; 10, lane 2; 20, lane 3; 30, lane 4; and 50, lane 5 units/ml. Lane 2 shows results with the probe digested with 10 units/ml DNase I in the absence of nuclear extract. Lanes A, C, G, and T are the sequence ladder of the same labeled DNA probe. The sequence to the right of lane 5 represents the nucleotides of the protected fragment.

To determine whether the observed footprint was the result of a specific protein-DNA complex formation, we carried out gel mobility experiments using a double-stranded oligonucleotide corresponding to sequences −440 to −470. The oligonucleotide was labeled with [32P]ATP (using the 5′ end labeling technique) and incubated with the nuclear extract from BAE cells. DNA–nuclear extract complexes were analyzed by gel electrophoresis in a 5% nondenaturing polyacrylamide gel. The results demonstrated that the oligonucleotide corresponding to sequences −440 to −470 interacted with a nuclear factor(s) (Fig. 2). This complex was shown to be specifically abolished by the presence of a specific competitor (an excess of unlabeled oligonucleotides corresponding to the same region). Nonspecific competitors (an excess of unlabeled oligonucleotides corresponding to the sequences −320 to −350 in the negative region) did not abolish the formation of this complex (Fig. 2). Oligonucleotides corresponding to other sequences of the 200 bp of the negative regulatory region did not form a specific complex when used as probes in gel mobility experiments (data not shown). These results were consistent with the hypothesis that a cis-acting element in the negative regulatory region interacts with a possible “repressor” protein. They also indicated that this element was located in the region spanning nucleotides −470 to −440 of the vWF promoter.

FIG. 1. Footprint analysis of the 5′-negative regulatory region of the vWF promoter. The DNA probe fragment corresponding to the −550 to −320 nucleotide sequences of the vWF gene was generated by PCR. Primers used were a 5′ end-labeled primer that corresponded to sequences −320 to −350, and an unlabeled primer that corresponded to nucleotide sequences −500 to −550. The DNA probe (10,000 cpm) was incubated with nuclear extract (30 μg) from BAE cells for 15 min on ice and digested with DNase I enzyme: 0, lane 1; 10, lane 2; 20, lane 3; 30, lane 4; and 50, lane 5 units/ml. Lane 2 shows results with the probe digested with 10 units/ml DNase I in the absence of nuclear extract. Lanes A, C, G, and T are the sequence ladder of the same labeled DNA probe. The sequence to the right of lane 5 represents the nucleotides of the protected fragment.

FIG. 2. Gel mobility experiments using oligonucleotides from the negative regulatory region of the vWF promoter. Nuclear extracts (4 μg) from BAE cells were incubated with 5′ end-labeled oligonucleotide probes (8000 cpm). Probes corresponded to sequences −320 to −350 (probe P, left three lanes), and sequences −440 to −470 (probe V, right three lanes) of the vWF negative regulatory region. The arrow shows the position of the specific complex. The free probes are shown at the bottom of the gel. The competitors were a 100-fold excess of unlabeled oligonucleotides (P′ (−350 to −320) or V′ (−470 to −440)). The incubation reactions were carried out as described under “Materials and Methods,” and samples were analyzed on a 5% nondenaturing polyacrylamide gel.
von Willebrand Factor Promoter Repression by NF1

Fig. 3. Gel mobility experiments indicating that the mutant oligonucleotides of the vWF promoter do not form specific protein-DNA complex. Gel mobility assays were carried out using 5'-end-labeled probes and BAE nuclear extracts as described in legend to Fig. 2. A, sequences of the wild type and each of four mutant oligonucleotides are shown. The 3-bp substitutions in each mutant oligonucleotide are indicated by bold letters. B, the probe (V) used in all samples is the wild type oligonucleotide spanning sequences in the region -470 to -440. The competitors used were a 100-fold excess of unlabeled oligonucleotides, corresponding to -440 to -470 (V), -320 to -350 (N), and the mutant -440 to -470 oligonucleotides (m1, m2, m3 and m4, respectively) (shown in A). C, the gel mobility experiment was carried out with wild type (V), and each mutant oligonucleotide (m1, m2, m3, and m4) was used as probes. The arrow shows the position of the specific complex.

because we did not observe the same complex in any of the other mutants m2, m3, or m4. Base changes corresponding to the m1 mutation were not used to generate vWF promoter fragments containing mutation in the repressor binding site. These mutation analyses establish that the entire intact -470 to -440 sequence was necessary for binding of the newly identified protein(s) to this region.

The Negative Regulatory Element in the Sequence -440 to -470 of the vWF Promoter Interacts with an NF1-like Protein—To determine the identity of the protein(s) that interacted with the -440 to -470 bp region, we analyzed the nucleotide sequences in this region and identified a DNA sequence that had homology with the binding site for the NF1 protein. The consensus NF1 binding site in this region and identified a DNA sequence that had homology with the binding site for the NF1 protein (Fig. 3A). Gel mobility experiments were carried out with BAE nuclear extracts using the vWF -440 to -470 oligonucleotide (V) as a probe. An oligonucleotide that corresponded to NF1-binding sequences of the adenovirus origin of replication (A) (23, 29) was used as a competitor. Formation of a DNA-protein complex with the probe V was abolished in the presence of a 100 × molar excess of the unlabeled adenovirus NF1 binding oligonucleotide designated as “A” (Fig. 4A, lane 4). An adenovirus oligonucleotide that contained a mutation in the NF1 binding site (mA) did not abolish the formation of this complex (Fig. 4A, lane 5). When the adenovirus NF1 binding oligonucleotide (A) was used as a probe, the specific NF1-DNA complex that formed migrated to the same position as that observed with vWF sequences (Fig. 4A, compare lanes 1 and 6). Furthermore, addition of a 100 × molar excess of unlabeled -440 to -470 oligonucleotide (V) as a competitor abolished the formation of the specific NF1-DNA complex (Fig. 4A, lane 9, and Fig. 4B, lane 2). Addition of a 100 × molar excess of unlabeled vWF oligonucleotide (mA) that contained the mutation in the NF1 binding site did not abolish the formation of this complex (Fig. 4B, lane 3). These results establish that the protein(s) interacting with sequences -440 to -470 of the negative regulatory region of the vWF promoter include an NF1-like protein.

An NF1-like Protein Functions as a Repressor of vWF Transcription in Endothelial Cells—Mutation analyses and transfection studies were next carried out to determine whether the NF1-like factor that interacted with the sequences -440 to -470 functioned as a repressor of vWF promoter activity. Constructs of the vWF promoter containing triple base substitutions in this region were fused to the growth hormone structural gene (used as a reporter gene) for these analyses. The
triple base substitutions were the same as that in oligonucleotide m3, which was shown to inhibit the DNA-protein interaction in gel mobility experiments (Fig. 3). We incorporated this mutation in the context of the promoter that included the core region and the negative regulatory region (sequences 2487 to 1155) to generate plasmid HGH-Brm3 (Fig. 5A). The plasmid containing the wild type sequences was designated HGH-B (22). Each of the plasmids was transfected into BAE cells, and the promoter activity of the mutated vWF sequences (HGH-Brm3) was compared with that of the wild type (HGH-B) and to that of the plasmid containing only the core promoter region (HGH-1).

The results of growth hormone production assays indicated that the promoter activity of the mutant HGH-Brm3 was similar to that of HGH-1 (Fig. 5A). The negative regulatory region of the unaltered vWF promoter inhibited the activity of the core promoter (Fig. 5, compare HGH-1 and HGH-B). These results indicated that the mutation in the NF1 binding site abolished its inhibitory effect in the negative regulatory region of the vWF gene promoter in BAE cells.

Previous results showed that the basal level of growth hormone expressed from the plasmid HGH-B was not due to vWF
control for transfection efficiency, plasmid protection analyses were carried out with this plasmid. As a comparison, transcription from the correct initiation site, similar RNase protection analyses demonstrated that the absence of detectable vWf transcript from plasmid HGH-B, whereas the vWf transcript was detected from plasmid HGH-Brm3 (Fig. 5). The results of the RNase protection analysis confirmed that there was no detectable vWf transcript from the HGH-B plasmid (Fig. 5B).

To confirm that the increase in the level of growth hormone expression in plasmid HGH-Brm3 correlated with an increase in transcription from the correct initiation site, similar RNase protection analyses were carried out with this plasmid. As a control for transfection efficiency, plasmid 5SVHPβWT (25) containing the human globin gene was cotransfected with each of the vWF-HGH plasmids. Results of the RNase protection experiments confirmed that there was no detectable vWf transcript from plasmid HGH-B, whereas the vWf transcript was detected from plasmid HGH-Brm3 (Fig. 5B).

These results demonstrated that mutation in sequences 470 to 440 of the vWF promoter inhibited protein-DNA complex formation and also abolished the inhibitory effect of the negative regulatory region restoring transcription. The data establish that the NF1-like protein that interacts with sequences 470 to 440 bp functions as a repressor of vWF transcription in endothelial cells.

An NF1-like Protein Functions as a Repressor of vWF Transcription in Nonendothelial Cells—The negative regulatory region of the vWF promoter was shown to have an inhibitory function in both vWF expressing and nonexpressing cell types. To determine whether the same repressor was present in nonendothelial cells, nuclear extracts were made from BSM cells and used in gel mobility experiments with the radioactively labeled oligonucleotide 470 to 440. The results of this analysis (Fig. 6) indicated that the BSM nuclear extract formed a specific complex with the DNA probe which migrated in the same position as the complex formed with the BAE nuclear extract. The formation of this complex was also abolished in the presence of a specific competitor, whereas the nonspecific competitor had no effect (Fig. 6). These results were consistent with the hypothesis that a repressor protein (identified as an NF1-like protein in BAE cells) was present in both vWF expressing and nonexpressing cell types and interacted with the negative regulatory region.

To determine whether a mutation in the NF1 binding site also abolished the repression of the promoter activity in nonendothelial cells, transfection experiments were next carried out in bovine smooth muscle cells. When plasmids HGH-B and HGH-Brm3 were transfected into BSM cells, similar results to those observed in endothelial cells were obtained (Fig. 7). The level of growth hormone expression from plasmid HGH-B was reduced compared with that of HGH-1, whereas the level of expression from plasmid HGH-Brm3 was comparable with that of HGH-1 (Fig. 7). These results establish that the NF1-like protein functions as a repressor of vWF transcription in nonendothelial cells as well as endothelial cells.

Mutation of the NF1 Binding Site Is Not Sufficient to Over-ride Endothelial Cell-type-specific Activity of Sequences 487 to 247 of the vWF Promoter—Sequences spanning nucleotides 155 to 247 in the 3′-terminal region of the first exon of the vWF promoter have been reported to abolish the inhibitory effect of the negative regulatory region only in endothelial cells (22). To determine whether the endothelial cell-specific activity of sequences 487 to 247 bp of the vWF promoter was dependent only on binding of the NF1-like protein to the negative regulatory region, we next tested the effect of the same 3 bp substitutions when incorporated into the context of the larger promoter fragment 487 to 247 to generate plasmid HGH-Krm3 (this construct is the same as wild type plasmid HGH-K except for the 3 bp substitution). The activities of the mutated and wild type plasmids were then analyzed in transfected BAE and BSM cells. The results (Fig. 8) demonstrated that both plasmids facilitated a similar level of growth hormone expression in BAE cells. This result was consistent with the hypothesis that the positive regulatory region overcomes the effect of the repressor in BAE cells. Thus, mutation of the NF1 binding site had no additional effect. However in BSM cells, both mutated and wild type plasmids behaved similarly, and there was no increased activity as a result of mutation in the NF1 binding site (Fig. 8).

The present results indicate that inhibition of the repressor function of the NF1 protein was not sufficient to abolish cell-type-specific activity of sequences 470 to 247 (a larger fragment) of the vWF promoter. Other DNA cis-acting elements (in addition to the NF1 binding site) within the vWF promoter region must also be able to repress promoter activity in nonendothelial cells. The presence of sequences 155 to 247 bp was the only difference between the active HGH-Brm3 and inactive HGH-Krm3. Thus, comparison of the activity of these two plasmids (both containing the NF1 binding site mutation) in BSM cells indicated that other repressor element(s) may be located in the sequences spanning 155 to 247 bp of the vWF gene.

DISCUSSION

We have previously demonstrated that the region of the vWF promoter spanning nucleotides 487 to 247 functions in an endothelial cell-specific manner. This region was shown to consist of a core promoter, a negative regulatory region, and a positive regulatory region. The core promoter that constitutes nucleotides 90 to 22 bp was shown to drive the expression of a heterologous human growth hormone gene in all cell types studied. Addition of sequences upstream of nucleotide 90 that spanned the region 312 to 487 bp was shown to inhibit core promoter activity in all cell types studied. Sequences corresponding to nucleotides 155 to 247 of the first exon were shown to counteract the inhibitory effect of the negative region in an endothelial cell-specific manner.

We have demonstrated the presence of several cis-acting
Our present data demonstrate that the inhibitory function of the upstream negative regulatory region of the vWf promoter was mediated through the interaction of an NF1-like protein with nucleotides spanning sequences −440 to −470. Three-bp substitutions that inhibited the interaction of the NF1-like protein with these sequences abolished the repressor function of the negative regulatory region. The NF1 protein was originally characterized as a 47-kDa protein that interacted with sequences at adenovirus origin of replication and was necessary for viral DNA replication (23, 29). However, DNA sequences that comprise the negative regulatory region of a number of genes including human retinal binding protein (30), chicken β-globin (31), mouse Ren-1d (32), Sparc (33), collagen (34), and rat growth hormone (19) are reported to have homology to the NF1 binding sequence. Proteins that recognize NF1 binding sequences are part of a family of proteins and are designated "NF1-like proteins" (19). In addition, other proteins such as C/EBP (35, 36), CP1, and CP2 (37) and a subset of modified histone H1 (38) also can interact with the NF1 binding DNA sequences.

To our knowledge there are no antibodies available that recognize NF1 proteins. Thus, we were not able to directly demonstrate by supershift that the specific protein that interacts with sequences −470 to −440 is the previously reported NF1 protein. However, the binding characteristics of our identified protein were similar to members of the NF1 family. The DNaseI footprint analyses demonstrated the protection of an approximately 24-nucleotide region, and mutation analyses demonstrated that the entire 30 nucleotides in the −440 to −470 region were necessary for DNA-protein interaction. These results were consistent with previously reported characteristics of the NF1-DNA interaction (23). Furthermore, gel mobility experiments demonstrated competition of vWf sequences for the protein that binds adenovirus NF1 binding sequences, indicating that the protein(s) recognizing adenovirus NF1 binding site and the vWf sequences are members of the NF1-like protein family.

A direct role for an NF1-like protein as a repressor has been previously reported (19). A 52-kDa protein member of this family was shown to function as a repressor of rat growth hormone promoter in nonpituitary cells (19). This result demonstrated that an NF1-like protein functioned as a repressor that was involved in cell-type-specific gene regulation. Our results establish that an NF1-like protein also functioned as a repressor of the vWf promoter, but it inhibited the vWf promoter activity in both endothelial and nonendothelial cell types.

There are several possible mechanisms that might describe how a repressor functions (15, 16). A repressor may inhibit promoter activity by interfering with the function of an adjacent activator. This inhibition may be achieved either through protein interaction when both activator and repressor are bound to DNA or through repressor binding to the DNA which may mask the binding site of an activator. In each of these mechanisms, the repressor interferes with the function of an activator that is required to interact with general transcription machinery to initiate transcription. Another mechanism by which a repressor might function is proposed to be through direct interaction with the components of general transcription machinery, thereby preventing transcription.

Although we do not have direct evidence to demonstrate which mechanism is involved in repression of the vWf pro-
moter by NF1, our results favor a mechanism that involves direct repression. We reasoned that if there was an activator adjacent to the NF1 binding site, which was necessary for transcription, deletion of sequences from –487 to –90 bp should have abolished promoter activity. Such a deletion would have been expected to have removed both the NF1 and the putative activator binding sites. The observed transcriptional activity of the plasmid HGH-1 that did not include these sequences indicated to us that removal of these sequences did not abolish transcription. We cannot rule out that an activator(s) may bind to sequences downstream of the –90 nucleotide and that its function could have been inhibited by the NF1 repressor.

Although it was clearly demonstrated that the NF1-like protein functioned as a repressor of the vWF promoter, mutation of the NF1 binding site in the context of the larger endothelial cell-specific vWF promoter fragment (–487 to +247) did not result in promoter activation in bovine smooth muscle cells. The same mutation in the context of the promoter that included the smaller core and negative regulatory region (–487 to +155) resulted in promoter activity in smooth muscle cells. These results suggested that sequences spanning nucleotide +155 to +247 of the vWF promoter inhibit promoter activity in nonendothelial cells even when the NF1 binding site is mutated. Thus, another repressor may interact with sequences in this region and inhibit expression in nonendothelial cells. Since this same +155 to +247 region positively activated vWF expression in endothelial cells, we hypothesize that a second repressor functions in a cell-type-specific manner and represses vWF promoter activity only in nonendothelial cells. However, this hypothesis does not exclude the possibility that this repressor may also be present in endothelial cells with its function inhibited (similar to that of NF1) by positively activating factors such as GATA factor (and perhaps another as yet unidentified factor) in endothelial cells.

The results of our deletion and mutation analyses of the vWF promoter are summarized in Fig. 9. We propose a model to explain the endothelial cell-type-specific regulation of the vWF gene, through a mechanism involving selective "de-repression" (Fig. 9). Such a mechanism involves the presence of factors that override the repressor function of the NF1-like protein (and perhaps the second downstream repressor) on the vWF pro-
moter in endothelial cells. The observation that a positive regulatory region is necessary for activation of the vWF promoter in endothelial cells suggested that trans-acting factors may interact with this region and play a role in de-repression. We have already demonstrated that mutation of the GATA binding site abolishes the promoter activity of sequences −487 to +247 in endothelial cells. Thus, the GATA factor (shown as G in Fig. 9B) and perhaps other as yet unidentified factors (represented as A? in Fig. 9) that interact with sequences +155 to +247 may function as de-repressors. The observation in smooth muscle cells that the sequences +155 to +247 inhibit the GATA promoter activation even in absence of NF1 binding (as shown by lack of expression of plasmid HGH-Krm3 in BSMC, Fig. 8) suggests that this second repressor may also directly inhibit transcription rather than interfere with the de-repression function of an activator. Thus, the presence of two independent repressors may be responsible for preventing the expression of the vWF gene in cells that are not of endothelial origin.

Studies with transgenic mice expressing the endothelial cell-specific vWF promoter have demonstrated that the sequences −487 to +247 of the vWF promoter function in an organ-specific manner (39). In mouse embryos this promoter was active in the endothelial cells of most of the embryonic vasculature, whereas in adult mice its activity was restricted to a subset of brain endothelial cells (39). It has been demonstrated that endothelial cells are heterogeneous in vivo, and that of different organs express different molecules. Most endothelial cells do express vWF (6). Thus, the organ-specific activity of the −487 to +247 region suggests that there are other cis-acting elements that are necessary to activate the vWF promoter in endothelial cells of other organs in vivo. This organ-specific behavior of the vWF promoter may be modulated through the NF1-like protein. Selective de-repression of the vWF promoter may be extended to include cis-acting elements in other regions of the gene that are required to overcome NF1 repression in endothelial cells of various organs, whereas elements in the terminal region of the first exon are sufficient to override the repression in endothelial cells of the brain vasculature. A secondary repressor of the vWF promoter in the +155 to +247 region may function to maintain repression of vWF transcription in cells of nonendothelial origin.

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