

The NFY transcription factor functions as a repressor and activator of the von Willebrand factor promoter

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Human von Willebrand factor (VWF) gene sequences -487 to $+247$ function as an endothelial-specific promoter *in vitro*. Analysis of the activation mechanism of the VWF promoter has resulted in the identification of a number of cis-acting elements and trans-acting factors that regulate its activity. The GATA and Ets transcription factors were shown to function as activators of transcription, whereas NF1 and Oct1 were shown to repress transcription. We have reported the presence of another repressor element in exon 1 that interacted with a protein complex designated "R." In the absence of NF1 binding, inhibition of this interaction resulted in promoter activa-

tion in nonendothelial cells. We have now identified the "R" protein complex as the NFY transcription factor. Using DNA methylation interference assay and base substitution mutation analysis, we show that NFY interacts with a novel DNA sequence corresponding to nucleotides $+226$ to $+234$ in the VWF promoter that does not conform to the consensus NFY binding sequence CCAAT. The VWF gene does contain a CCAAT element that is located downstream of the TATA box and we show that the NFY factor also interacts with this CCAAT element. Using antibodies specific against the A, B, and C subunits of NFY, we demonstrate that the NFY complexes interacting with the

CCAAT sequence have a composition similar to that of the repressor binding to the first exon sequences. The results of mutation analysis and transfection studies demonstrated that the interaction of NFY with the upstream CCAAT element is required for VWF promoter activation. Based on these results, we hypothesize that NFY can function both as a repressor and activator of transcription and its function may be modulated through its DNA binding sequences. (*Blood*. 2002;99:2408-2417)

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Introduction

Endothelial cells form a monolayer that covers the surface of all blood vessels. These cells participate in many physiologic and pathophysiologic processes, and their accessible locations make them an attractive target for development of gene therapy approaches for the treatment of many diseases.¹⁻⁶ Interest in endothelial cells has resulted in an expanding body of research on the mechanism of endothelial-specific gene regulation. The goals of many of these studies are to first identify the regulatory regions of the endothelial-specific genes and then determine the molecular mechanism that governs the endothelial-specific activation of gene expression. Target genes for these studies include tie-1,⁷ tie-2,⁸ VEGF receptors 1 and 2,⁹⁻¹¹ endothelin,¹² PECAM-1,¹³ and VWF.¹⁴⁻¹⁶ The activation patterns of these promoters have been studied in cell culture, and for some promoters also in transgenic mice.^{8,14,15,17,18} Despite its limitations, *in vitro* analysis in cell culture provides a feasible approach toward determining the role of the multiple potential cis-acting elements involved in regulating promoter activation.

We have previously demonstrated that a 734-bp region of the VWF gene spanning sequences -487 to $+247$ activated gene expression in endothelial cells in culture.¹⁴ The highly endothelial cell-restricted activation pattern of this promoter region was confirmed later by 2 independent groups.^{6,19} Deletion and base substitution mutation analyses of the VWF promoter by others and

our group have resulted in the identification of several cis-acting elements and trans-acting factors that both positively and negatively regulate promoter activity. The GATA and Ets transcription factors function as activators,^{14,20} whereas Oct1 and NF1 function as repressors of VWF promoter activity.^{21,22}

We have also reported the presence of another repressor designated "R" that interacted with sequences $+215$ to $+247$ in the VWF promoter.²³ We demonstrated that while inhibition of either the NF1 or "R" protein complex's interaction with VWF promoter sequences was not sufficient to activate promoter function in nonendothelial cells, the simultaneous inhibition of the binding of both repressors was sufficient to do so.²³ The important role of repressors in regulating cell type-specific activity of the VWF promoter is also supported by the studies of Nettelbeck et al,¹⁹ which demonstrated a 20- and 169-fold enhancement of VWF promoter activity in bovine aortic endothelial (BAE) cells and human umbilical vein endothelial cells (HUVEC), respectively (by using a strategy that establishes a positive feedback loop involving simultaneous expression of a strong artificial transcriptional activator), without the loss of specificity.¹⁹ This highly maintained cell type-specific function of the VWF promoter was hypothesized to be due to the role of repressors that inhibit the VWF promoter activity in nonendothelial cells.¹⁹ We now report that the "R" repressor protein is the transcription factor NFY with a novel

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binding site that does not include the CCAAT sequence. We also report that NFY binds to a CCAAT element corresponding to sequences -18 to -14 in the VWF promoter, and in that context functions as an activator. Our results demonstrate a novel binding site for NFY and indicate that this transcription factor has a dual role (repressor and activator) in regulating VWF promoter activity that is specified through its binding sequence.

Materials and methods

Plasmid construction

Generation of plasmids HGH-K, HGH-1K, and HGH-1 were previously described.¹⁴ To generate the plasmid K-NFY, fragments -90 to +1 and -30 to +247 of the VWF promoter were first amplified by polymerase chain reaction (PCR). Specific 30 base oligonucleotide primers that corresponded to the 5' and 3' regions of each fragment were used to obtain 2 double-stranded fragments that contained the -30 to +1 sequences in common. The base substitutions TC (or AG) instead of AA (or TT) in the CCAAT sequence were incorporated into both -30 to +1 complementary primer sequences. The 2 mutant fragments were then combined and used as a PCR template to generate the -90 to +247 fragment containing the mutation in CCAAT element. For this second PCR, the primers that corresponded to -90 to -60 and +213 to +247 with an additional 9 bases that contained *Hind*III and *Sal*I sites, respectively, were used. The PCR and cloning of the mutant -90 to +247 sequences into P ϕ HGH were as previously described for the generation of the wild-type HGH-1K fragment.¹⁴ This plasmid was then digested with *Hind*III and ligated to a fragment corresponding to sequences -487 to -90 of the VWF gene that was obtained from *Hind*III digestion of the HGH-K plasmid. This resulted in generation of the K-NFY plasmid that contained sequences -487 to +247 of the VWF promoter with mutation in the CCAAT element. To generate the plasmids HGH-KY and HGH-KRY, fragments corresponding to sequences -487 to +247 were amplified using primers that corresponded to sequences +217 to +247 with base substitution mutations at +226 to +228 (CCG to GAA) and a *Sal*I site at the 3', and wild-type sequence -487 to -433 (for generation of HGH-KY) or sequence -487 to -433 with base substitution mutations at -445 to -443 (GAT to TCG, for

generation of HGH-KRY), which was the same mutation in the NF1 binding site as previously described.²² Both these primers also contained *Sal*I sites at their 5' end. Generated mutant fragments were cloned into the *Sal*I site of P ϕ HGH as previously described.¹⁴ Plasmid HGH-1KY was generated by digestion of plasmid HGH-KY with *Hind*III restriction enzyme, removal of VWF sequences corresponding to -487 to -90 and religation of the remaining plasmid sequences as previously described for generation of HGH-1K plasmid.¹⁴ Sequences of all plasmids were confirmed by dideoxy sequencing method.

Cell culture and transfection

Primary BAE cells (passage 1) were obtained from Clonetic (San Diego, CA) and maintained as previously described.¹⁴ BSM cells were maintained as previously described.¹⁴ HeLa and HEK 293 cells were maintained as previously described for HeLa cells.¹⁴ Stable transfections of BAE and HEK293 cells were carried out using the calcium phosphate precipitation technique as previously described.^{23,24} For stable transfections, BAE and HEK293 cells were cotransfected with plasmid mixtures that contained the neomycin resistance gene under the control of the RSV promoter (2 μ g), and the various VWF-HGH plasmids (20 μ g). Growth hormone assay was carried out using the transient growth hormone assay system (Allegra; Nichols Institute Diagnostics, San Juan, CA) in the first 48 hours posttransfection and then after selection in neomycin drug as previously described.^{23,24}

Statistical analyses

The statistical analyses were carried out using the Student *t* test (2 samples assuming unequal variances).

Gel mobility and supershift assays

Nuclear extracts from cells were prepared by the method of Schreiber et al.²⁵ Oligonucleotides corresponding to sequences +215 to +247 (sequence shown by dotted line in Figure 1) and -30 to +1 of the VWF promoter were radioactively labeled using 32P- γ ATP and polynucleotide kinase. The oligonucleotide probes were incubated with nuclear extracts (5 μ g) in the presence or absence of competitors in a 20- μ L reaction mixture containing 100 mM KCl, 10 mM HEPES (pH 7.9), 2.5 mM MgCl₂, 0.5 mM

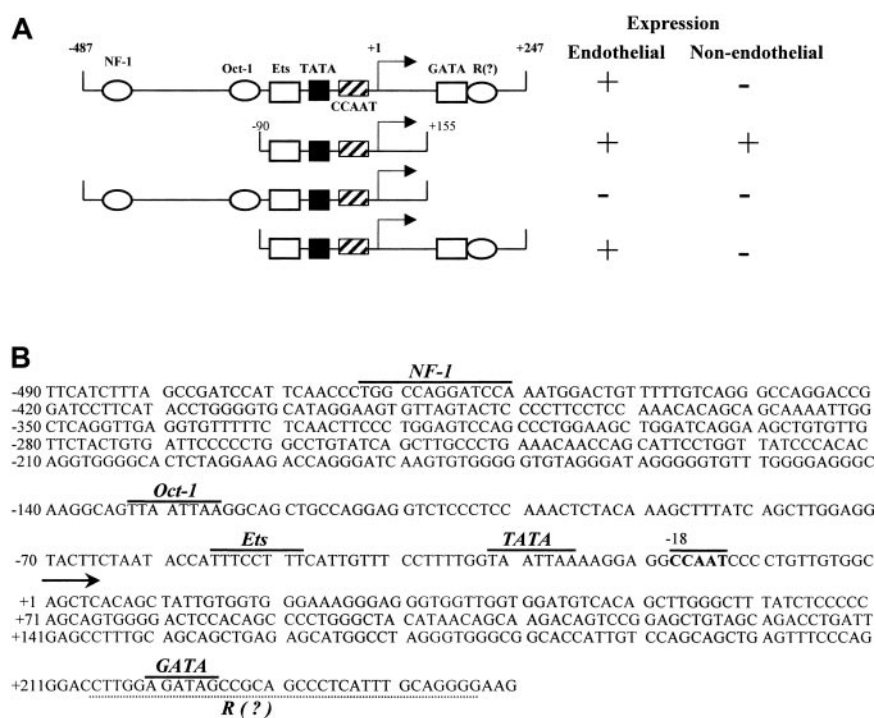


Figure 1. Schematic representation and nucleotide sequence of the VWF promoter. (A) A schematic representation of the VWF promoter and the position of cis-acting elements and transacting factors that bind them are shown. The factors that function as repressors (NF1, Oct-1, and R?) are represented by circles and the factors that function as activators (Ets and GATA) are represented by rectangles. The TATA and CCAAT sequences are shown by filled and hatched rectangles, respectively. The arrow represents the position of the transcription initiation site. The expression pattern of various fragments of the promoter in endothelial and nonendothelial cells are shown. The + and - represent the promoter activity and lack of activity, respectively. (?) indicates unidentified. (B) The nucleotide sequence of the VWF promoter with the exact position of cis-acting elements is shown. The dotted line represents the sequence +215 to +247 that was shown to interact with protein complex "R."

dithiothreitol, and 1 μg poly (dI-dC) for 30 minutes at room temperature. The competitors were wild-type or mutant oligonucleotides that correspond to VWF sequences +215 to +247, oligonucleotides containing E2A binding site 5'GAACCAGAACACCTGCAGCA3', C/EBP binding site 5'TGCAGATTGCGCAATCTGCA3', and NFY binding site 5'CGTCTC-CACCAATGGGAGGGCTGGGC3'. For gel mobility supershift assays, extracts were preincubated with antibodies (1 μg) under the conditions described above for 3 hours at 4°C prior to the addition of the labeled probes and then further incubated for 15 minutes at room temperature following addition of the probes. Antibodies used in the supershift assays were mouse monoclonal anti-NF-YA antibody (Pharmingen, San Diego, CA), rabbit polyclonal anti-NF-YB and anti-NF-YC antibodies (gift of R Mantovani), monoclonal anti-E2A, TAL1, and rabbit polyclonal anti IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA). All protein-DNA complexes were resolved on a 5% nondenaturing polyacrylamide gel in 1X Tris-borate ethylenediaminetetraacetic acid (EDTA) buffer. Gels were dried and subjected to autoradiography. Densities of protein-DNA complexes were quantified by phosphorimager analysis using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

DNA methylation interference

DNA methylation interference was carried out according to Schwarzenbach²⁶ with slight modifications. Briefly, 2 DNA probes corresponding to sequences +215 to +247 of the VWF promoter were made by 32P-3' end labeling of either the top or bottom strands independently. The end-labeled DNA probes were partially methylated at the guanine residues by incubation for 5 minutes with dimethyl sulfate, followed by ethanol precipitation and resuspension in 10 mM Tris-HCl and 0.1 mM EDTA (pH 8.0) at 20 000 cpm/ μL . Methylated DNA probes (300 000 cpm) were used in binding reactions with HeLa cell nuclear extracts (120 μg) for 30 minutes, in a total reaction volume of 100 μL . The binding buffer and gel electrophoresis conditions were the same as described for the gel mobility assay. After electrophoresis, wet gels were exposed to x-ray autoradiography film overnight, and portions of the gel containing bound DNA ("X" and "NX" fractions) and unbound DNA ("F" fraction) were excised. The DNA was purified from the gel by electroelution onto a diethylaminoethyl membrane, and exposed to 1M piperidine at 90°C for 30 minutes. The piperidine-treated unbound and protein-bound DNA were analyzed by gel electrophoresis on a 20% polyacrylamide/urea sequencing gel and exposed for autoradiography. The sequence of DNA was known by comparing the unbound ("F") fraction of the top-strand DNA probe to that of the bottom strand, running in parallel on the same gel.

Results

NFY transcription factor is a component of the repressor protein complex "R" that interacts with VWF promoter sequences in the exon 1

Human VWF promoter sequences spanning nucleotides -487 to +247 function as an endothelial-specific promoter. Deletion analysis and specific base substitution mutations previously performed by others and us demonstrated the presence of several cis-acting elements that either activate or repress VWF promoter activity (Figure 1). Deletion analysis demonstrated that whereas VWF promoter sequences that span -90 to +155 are active in both endothelial and nonendothelial cells, sequences spanning -90 to +247 are active only in endothelial cells.^{21,23} This indicates that the +155 to +247 region of the VWF (that includes the GATA binding site) is required for endothelial cell-specific activation of the VWF promoter, but the same region also contains sequences that repress promoter activity in nonendothelial cells.

Our previous analysis of the VWF promoter +155 to +247 region for the presence of potential repressor element(s) demonstrated that a protein complex (present in both endothelial and

nonendothelial cells) designated "R" interacted with sequences +215 to +247 and functioned as a repressor.²³ To characterize the "R" protein complex, we searched for the presence of potential DNA cis-acting elements with homology to previously reported transcription factors' binding sequences. Our analysis demonstrated the presence of an E box that is a binding site for the E2A transcription factors and their partner SCL/Tal1.^{27,28} Thus, to determine whether E2A transcription factors were a component of the "R" complex, and whether they were differentially present in "R" complexes in endothelial compared with nonendothelial cells, we carried out gel mobility experiments with nuclear extracts prepared from HeLa cells, bovine aortic smooth muscle (BSM), and BAE cells.

For these analyses, nuclear extracts were incubated with the double-stranded oligonucleotide probe corresponding to VWF sequences +215 to +247, in the presence or absence of competitor oligonucleotide that contained the consensus E2A binding site. Unlabeled oligonucleotide sequence +215 to +247 (ds-R) was used as a specific competitor, and for negative controls we used double-stranded oligonucleotide competitors containing binding sites for NFY and C/EBP transcription factors. The results of these analyses demonstrated that nuclear extracts from all 3 cell types displayed a similar pattern of complex formation in the absence of competitors, although the intensity of the bands corresponding to the complexes varied among the cell types (Figure 2, lanes 2, 7, 12). The probe formed a specific slow migrating major complex (MC) with nuclear extracts from all cell types that were abolished in presence of specific competitors as previously reported²³ (Figure 2, lanes 3, 8, 13). We also observed 3 faster migrating complexes

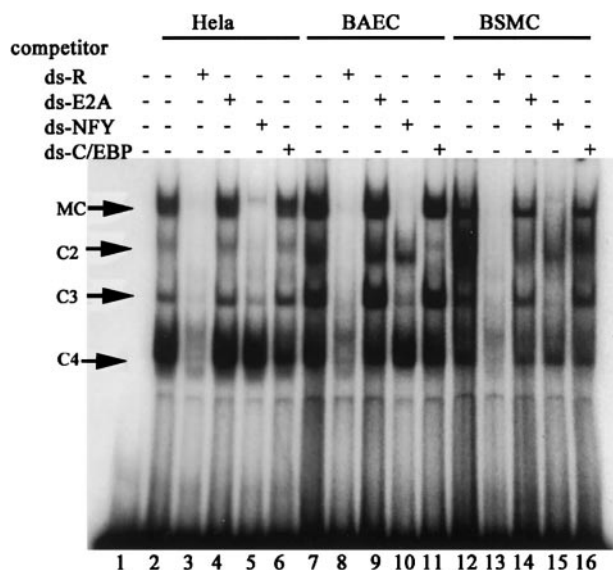


Figure 2. Inhibition of repressor protein complex interaction with VWF DNA sequences in the presence of NFY binding oligonucleotide competitor. Gel mobility experiments were carried out using a radioactively labeled oligonucleotide probe corresponding to DNA sequences +215 to +247 of the VWF gene. The probe (10 000 cpm) was incubated with nuclear extracts (5 μg) prepared from HeLa, BAE, and BSMC cells in the presence or absence of competitors and analyzed on a nondenaturing 5% polyacrylamide gel as described in "Materials and methods." The competitors were double-stranded oligonucleotides corresponding to sequences +215 to +247 (ds-R, specific competitor), and sequences that contained E2A, C/EBP, and NFY consensus binding sites. Lane 1 contains the probe alone in the absence of nuclear extracts. The - represents the absence and the + represents the presence of 100 \times excess of each competitor. Samples containing nuclear extracts (5 μg) from each cell type are shown under the bold lines. Arrows show the position of the MC and the 3 faster migrating minor complexes C2, C3, and C4. The free probe is shown at the bottom of the gel. The figure shown is a representative of 3 independent experiments.

(C2, C3, and C4) with varying intensities (depending on the cell types). However, the appearances of these complexes were not consistent in repeated experiments (eg, see Figure 3), suggesting that either these complexes were not specific or that the condition for their formation was not optimal in these experiments. Thus, the natures of these faster-migrating complexes were not pursued further. The competitor that contained the E2A consensus binding site did not abolish the formation of these specific complexes in any cell type, suggesting that E2A is not the component of the "R" protein that interacts with VWF sequences +215 to +247 (Figure 2, lanes 4, 9, 14). The competitor that contained the C/EBP consensus binding site also had no effect on the formation of the MC. However, the competitor that contained the NFY binding sequence inhibited the formation of the MC (as well as most of the other minor complexes) in all cell types (Figure 2, lanes 5, 10, 15). We have also used oligonucleotides that contain Tal1 and Oct binding consensus sequences and demonstrated that these oligonucleotide competitors did not inhibit the formation of any of these complexes (data not shown). These results suggested that the E2A transcription factors are not a constituent of the "R" protein complex that interact with VWF sequences +215 to +247, but the NFY transcription factor may be a component of this "R" complex.

To demonstrate directly whether NFY interacts with +215 to +247 sequences of the VWF promoter, supershift gel mobility experiments were carried out. In these assays, nuclear extracts from HeLa, BAE, and BSMC cells were preincubated with anti-NFY/A antibody, anti-E2A antibody, or an IgG antibody prior to the addition of the probe. The results demonstrated that the anti-NFY/A antibody was able to supershift completely the slow migrating MC in all 3 cell types, whereas no supershift was observed with either the IgG or anti-E2A antibodies (Figure 3).

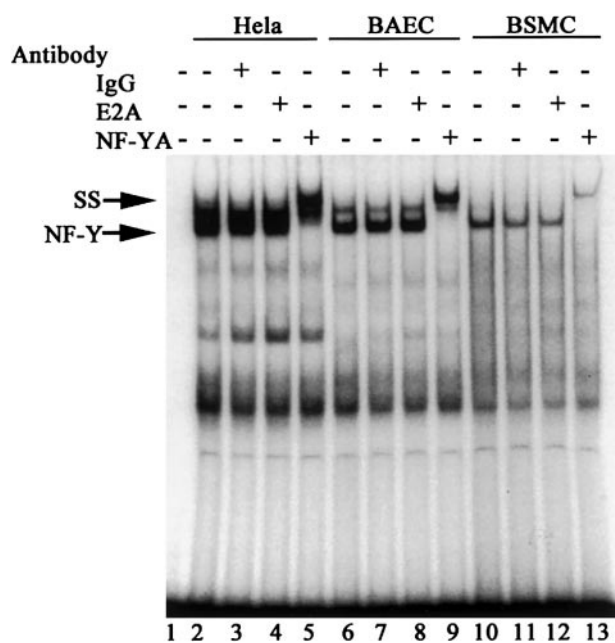


Figure 3. Identification of NFY as a component of protein complex "R." Nuclear extracts from HeLa, BAE, and BSMC cells (5 μ g) were incubated with IgG antibody, anti-E2A, or anti-NFY-A antibodies (1 μ g each) for 3 hours at 4°C prior to addition of the labeled probe. The probe used was the same as described in Figure 2. After addition of the labeled probe (10 000 cpm/reaction), reaction mixtures were incubated at room temperature for an additional 30 minutes and analyzed on a non-denaturing acrylamide gel as described in Figure 2. Lane 1 represents the probe alone with no nuclear extracts. Lanes 2 to 13 represent probe incubated with nuclear extracts in the presence (+) or absence (-) of each antibody. The positions of the NFY and supershifted complexes (shown as SS) are shown by the arrows. The figure shown is a representative of 2 independent experiments.

These results clearly demonstrate that an NFY transcription factor complex containing the NFY/A subunit interacts with sequences +215 to +247 of the VWF promoter.

Identification of the DNA sequences necessary for interaction of NFY with sequences +215 to +247 of the VWF gene

The consensus binding site for NFY is reported to be CCAAT.²⁹ Mutagenesis analyses by several laboratories have demonstrated that there is a strict requirement, with very few exceptions, for the presence of all 5 nucleotides to allow NFY binding.²⁹ However, sequence analyses of the +215 to +247 region of the VWF promoter that interacted with NFY did not demonstrate the presence of a CCAAT element. To identify the nucleotide sequences in this region that directly interacted with the NFY complex, we performed DNA methylation interference analysis. The double-stranded oligonucleotide corresponding to sequences +215 to +247 was labeled at one end, exposed to dimethyl sulfate, and incubated with HeLa cell nuclear extracts. The NFY-DNA complexes and the free probes were separated by acrylamide gel electrophoresis and eluted prior to cleavage by piperidine and analysis on polyacrylamide denaturing gels. Comparison of the sequence pattern of the free probe (Figure 4, lane 1), the probe bound to NFY that forms MC complex (Figure 4, lane 2), and the probe bound to the protein in C3 complex (Figure 4, lane 3) demonstrated the absence of 2 fragments corresponding to piperidine cleavage after the G nucleotides (on the bottom strand) at positions +232 and +233 specifically in the NFY-bound probe. The intensity of the band corresponding to cleavage after the G nucleotide (on the bottom strand) at position +236 was also significantly reduced compared with the unbound probe. However, comparison of the intensity of this band in the NFY-bound probe and the probe in the C3 complex suggests that this nucleotide may not be specifically involved in NFY-DNA interaction (Figure 4).

These results demonstrate that the G nucleotides at positions +232 and +233 on the noncoding strand are directly involved in the interaction with the NFY transcription factor. These data are consistent with our previous mutation analysis, which demonstrated that the 3 base substitution mutations of nucleotides +229, +232, and +233 abolished the "R" protein-DNA interaction.²³

However, these results do not exclude the possibility that additional sequences in the +215 to +247 region may also be required for NFY binding. To identify other potential DNA bases that may be involved in this interaction, we performed gel mobility competition assays. For these analyses, several double-stranded oligonucleotides corresponding to sequences +215 to +247 were generated; each contained a different 3 base substitution mutation as shown in Figure 5A. These mutant oligonucleotides were used as competitors (at concentrations 20 \times in excess of labeled probe) in gel mobility assays. When the wild-type sequence was used as a probe and the mutant oligonucleotides were present as competitors, the M1, M3, M5, and M6 oligonucleotides efficiently competed with the wild-type probe and inhibited NFY complex formation (Figure 5B). When these mutant oligonucleotides were labeled and used as probes directly, they formed an NFY-DNA complex (data not shown), thus indicating that base substitution mutations in these oligonucleotides do not effect NFY-DNA interaction. However, the M2, M4, and M7 oligonucleotides failed to compete with the wild-type probe and did not inhibit complex formation (Figure 5B). When these oligonucleotides were used at higher concentrations (100 \times excess), the M7 oligonucleotide was able to compete with the wild-type probe, whereas M2 and M4 did not (data not shown). In addition, when M2 and M4 oligonucleotides were labeled and

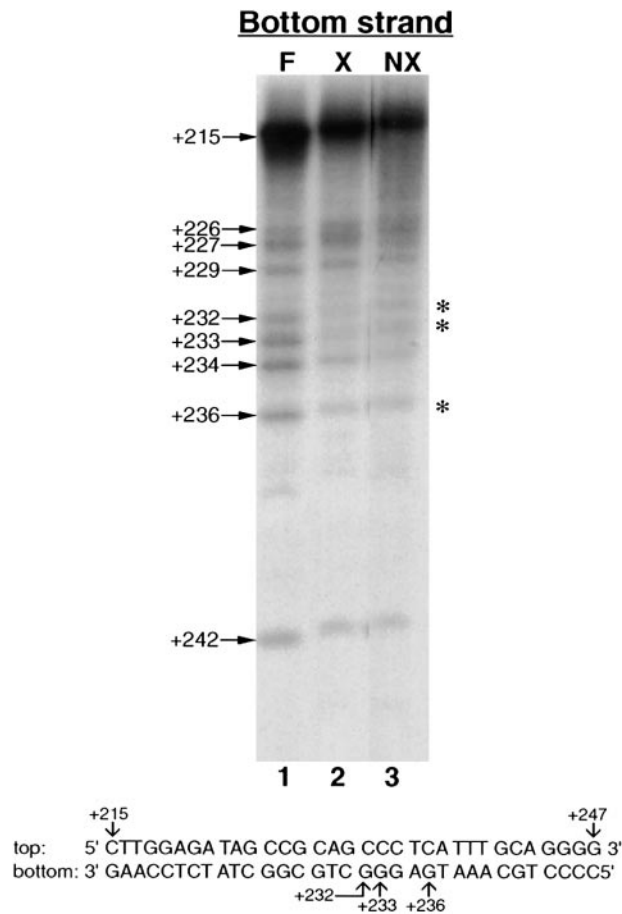


Figure 4. Identification of the G nucleotides in the repressor element that interact with NFY. Labeled DNA probe (bottom strand labeled) corresponding to sequences +215 to +247 was partially methylated with dimethyl sulfate, incubated with nuclear extracts, and complexes formed were resolved on a 5% nondenaturing acrylamide gel. NFY protein bound probe (X), probe bound to nuclear proteins to form complex C3 (NX), and free probes (F) were eluted and cleaved at modified bases with piperidine and resolved on 20% acrylamide sequencing gel. Positions of the G residues where methylation interfered with protein interaction are shown by asterisks. The numbers represent the position of G residues in the bottom strand that correspond to each band. The analysis of the protein DNA complex formed with the probe that was labeled at the top strand is not shown since no difference was observed in the pattern compared with that of free probe. The figure shown is a representative of 3 independent experiments.

used as probes directly, they failed to form an NFY-DNA complex (data not shown).

These results demonstrate that base substitution mutations in M2 and M4 oligonucleotides abolish NFY-DNA interaction, since these oligonucleotides did not compete with the wild-type probe when used as competitors (at either 20× or 100× excess), and did not form the NFY-DNA complex when used as probes. The base substitution mutations in the M4 oligonucleotide correspond to sequences +232, +233, and +234. This is consistent with the results of a previous mutation analysis²³ and methylation interference, which demonstrated the involvement of bases +232 and +233 in NFY-DNA interaction. The inhibition of NFY-DNA complex formation by base substitution mutation in M2 indicates that in addition to bases at +232 and +233, the CCG sequence at positions +226 to +228 is also necessary for efficient NFY-DNA interaction.

These data demonstrate that the NFY transcription factor interacts with a novel DNA element in the VWF gene promoter that constitutes nucleotides +226 CCGNNNCCC +234 and does not correspond to the previously reported CCAAT sequence.

In the absence of NF1 binding, mutation of the NFY binding sequence in the first exon results in VWF promoter activation in nonendothelial cells

Our previous analysis demonstrated that base substitution mutation in sequences +229, +231, and +232 in VWF promoter (in the absence of NF1 binding) resulted in VWF promoter activation in bovine smooth muscle cells,²³ thus functionally confirming that these base substitutions, which overlap with those shown by methylation interference and mutation analysis to be required for NFY binding, relieve inhibition of VWF promoter activity in smooth muscle cells. To further confirm the role of NFY as a repressor, we proceeded to determine the effect of mutations in the NFY binding sequence on VWF promoter activation in HEK293 as a model of a human nonendothelial cells. First, we confirmed the interaction of NFY with VWF sequences in gel mobility experiments using nuclear extracts prepared from HEK293 cells (data not shown). Next, we generated a mutant VWF promoter fragment that corresponded to sequences -90 to +247 with base substitutions in CCG element (in position +226 to +228), and we generated 2 other mutant VWF promoter fragments that corresponded to sequences -487 to +247, one containing the same base substitutions in CCG element alone and the other containing the same mutation in CCG element, in addition to base substitution mutations that abolish the upstream NF1-DNA interaction. Base substitutions in CCG were the same as the one described in Figure 5, which was shown to abolish NFY-DNA interaction. Base substitutions in the NF1 binding site were the same as those previously

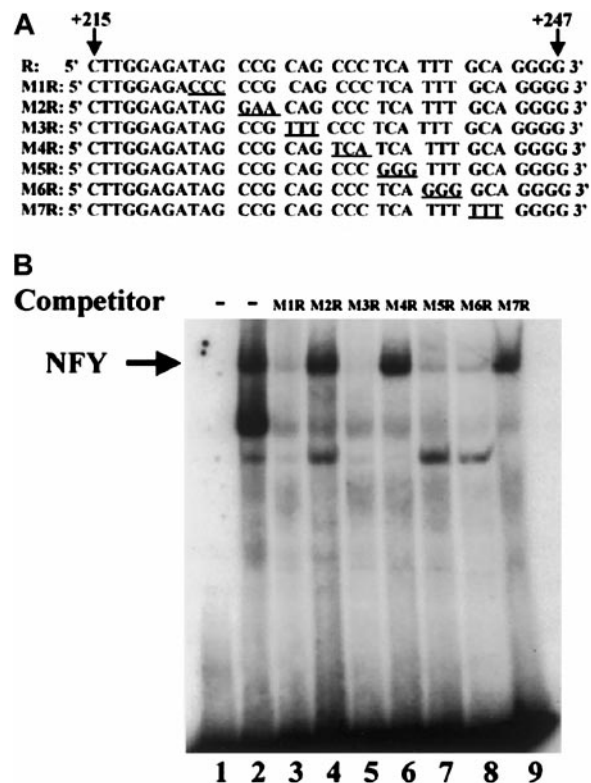


Figure 5. Characterization of the nucleotides in sequences +215 to +247 that are required for stable NFY-DNA interaction. (A) The sequences of the wild-type and mutant oligonucleotides corresponding to region +215 to +247 of the VWF gene are shown. The base substitution mutations in each oligonucleotide are underlined. (B) Gel mobility experiments were carried out using HeLa cell nuclear extracts as described in Figure 2. The probe used was the wild-type (R) oligonucleotide, and the competitors were mutant oligonucleotides shown in (A) and were present at 20× excess of the probe. The - represents the lack of competitors.

described.²² These mutant VWF promoter fragments were fused to a human growth hormone structural gene to generate plasmids HGH-1KY (containing VWF fragment -90 to +247 with mutation in CCG element), HGH-KY (containing VWF fragment -487 to +247 with mutation in CCG element), and HGH-KRY (containing VWF fragment -487 to +247 with double mutation in CCG element and NF1 binding site). We stably transfected HEK293 cells with these plasmids and those of the wild-type HGH-1, HGHG-1K, and HGH-K. Stable transfection analysis was carried out to generate conditions in which transfected plasmids could integrate into host cellular chromatin. This approach was based on previous reports on the function of NFY, which demonstrated that this transcription factor mediates nucleosomal assembly. Thus, to accurately determine the functional role of NFY in VWF promoter activation, plasmids may need to acquire chromatin structure. Our previous analysis of the "R" repressor function was also performed in stably transfected cells.²³

The level of growth hormone expressed in cells transfected with mutant and wild-type plasmids were determined as previously described.²³ The results (Figure 6A) demonstrated that the level of growth hormone expression from plasmids HGH-1K and HGH-K was significantly reduced (approximately 80%, $P < .01$) compared with that from HGH-1. However, the expression from HGH-1KY was similar to that from HGH-1 (5-fold increase in activity compared with wild-type HGH-1K, $P < .01$), and expression from HGH-KRY was significantly increased (2-fold, $P < .01$) compared

with that from HGH-K. We hypothesize that in the context of the -487 to +247 promoter, simultaneous mutation of the NF1 binding site and CCG element only result in partial activation of the promoter (compared with core promoter activity in HGH-1), due to the presence of an intact Oct 1 binding site, which also functions as a repressor.

These results demonstrated that in the absence of NF1 (and most likely Oct1) repressor binding, the base substitutions in the CCG element that inhibit NFY-DNA interaction relieve the repression of VWF promoter activation in nonendothelial cells.

Expression of these plasmids in BAE cells demonstrated that VWF promoter fragments corresponding to -90 to +247 (HGH-1K) and -487 to +247 (HGH-K) have a similar level of activity to that of the core promoter fragment (HGH-1) (Figure 6B). This was consistent with previous reports and confirms the endothelial cell type-specific activation of these promoter fragments. Also, these results support the hypothesis that the function of repressors is inhibited when these wild-type VWF promoter fragments are expressed in endothelial cells. The expression of mutant VWF promoters (containing mutations in NF1, NFY, or both binding sites) in BAE cells demonstrated a similar level of promoter activity to those of the wild type (Figure 6B), thus demonstrating that the mutation of NFY binding site neither significantly increased nor significantly decreased promoter activity in endothelial cells.

These results demonstrate that NFY (when binding to the downstream CCG element) did not function as an activator in endothelial cells. However, its function as a repressor in endothelial cells could not be directly demonstrated since the wild-type promoter fragments HGH-1K and HGH-K were as active as the core promoter (HGH-1) in the BAE cells.

Interaction of NFY with the upstream CCAAT element results in activation of the VWF promoter

The CCAAT consensus binding site for NFY is usually located approximately 60 bp to 100 bp upstream of the transcription start site in many eukaryotic genes, although the presence of a CCAAT element at an unusual position (-18 to -14) downstream of the TATA box in the VWF gene is reported.³⁰ We have recently demonstrated that NFY interacts with this CCAAT element and through this interaction mediates irradiation induction of the VWF core promoter sequences -90 to +22.³¹ In order to determine the role of the NFY interaction with this CCAAT element in the regulation of the endothelial-specific VWF promoter fragment (sequences -487 to +247), we carried out mutation and transfection analyses as described for the downstream repressor NFY binding site. For these analyses, a mutation in the CCAAT element that had previously been shown to inhibit the NFY-DNA interaction³¹ was incorporated into the sequence -487 to +247, and the resultant DNA fragment was fused to the human growth hormone structural gene to generate the plasmid K-NFY. Expression from the mutant VWF promoter was compared with that of the wild-type plasmid HGH-K in stably transfected BAE cells by determining the level of secreted growth hormone. The results demonstrate that the mutation of the CCAAT element significantly reduces the VWF promoter activity in BAE cells (Figure 7). We did not perform similar transfection analysis in BSM cells since the wild-type fragment is not expressed in BSM cells, thus no further information would be gained by expressing a mutant promoter in which mutation results in loss of function in expressing cell types.

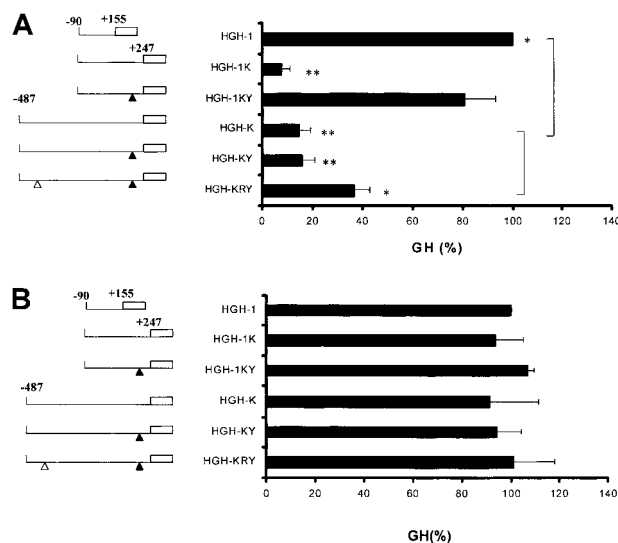


Figure 6. Mutation of the novel NFY binding element (R) activates the VWF promoter in nonendothelial cells. A schematic representation of the VWF-HGH plasmids containing the wild-type and mutant VWF promoter fragments is shown in the left. Solid triangles represent the base substitutions of CCG (nucleotides +226 to +228) in the novel NFY binding site. The open triangle represents the base substitution mutation in upstream NF1 binding site. HEK293 (A) and BAE (B) cells were stably transfected with a mixture of each VWF-HGH test plasmid and a plasmid containing the neomycin resistance gene. Transfected cells were continuously grown in presence of neomycin analogue G418 to select for stably transfected cells. Individual colonies of transfected cells were selected and growth hormone expression from each colony was determined and normalized to the cell number. The average level of growth hormone expression from HGH-1 plasmid in BAE and HEK293 cells was 25 ng/mL per 100 000 and 18 ng/mL per 100 000 cells, respectively, and the level of expression from other plasmids is shown as percentages of these levels in each graph. The results shown are the average of 12 to 15 colonies from 2 independent transfections for each plasmid and the error bar represents the standard deviation. The P values were more than .05 for transfections in BAE cells, which demonstrates no statistical significance in level of expressions from various plasmids in this cell type. The P values were less than .01 where indicated by double asterisk for transfection in HEK293. The bracket in graph A represents the plasmid activities that were compared for statistical analysis.

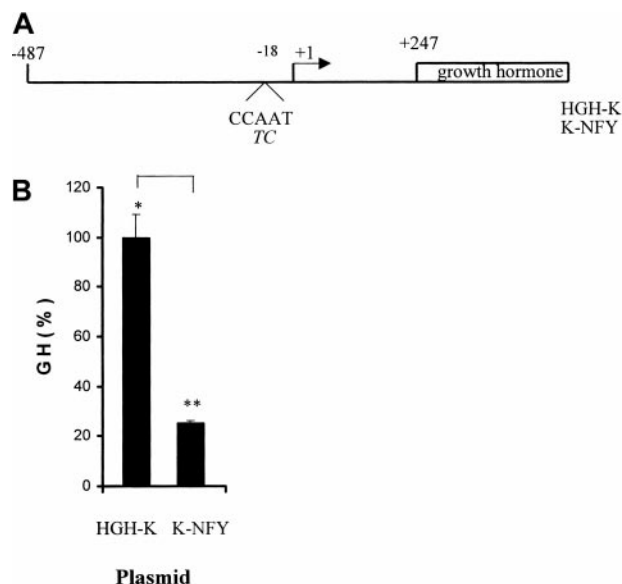


Figure 7. Reduction of VWF promoter activity by mutation of the CCAAT element. (A) A schematic representation of the plasmids HGH-K and K-NFY, with the base substitutions in the CCAAT element shown in italics. (B) BAE cells were stably transfected with a mixture of each VWF-HGH test plasmid and a plasmid containing the neomycin resistance gene. Transfected cells were continuously grown in the presence of the neomycin analogue G418 to select for stably transfected cells, and cells in the culture dish were allowed to reach confluency to obtain a heterogeneous population of stably transfected cells. Growth hormone expression from confluent plates was determined and normalized to the cell number. The results shown are the average of 2 independent transfections for each plasmid and the error bar represents the standard deviation. The statistical analysis demonstrated that the *P* value was less than .01. The average level of growth hormone expression from HGH-K plasmid in heterogeneous cell populations was determined to be 60 ng/mL per 100 000 cells.

These results demonstrate that NFY functions as an activator of the VWF promoter when it interacts with its consensus binding sequence CCAAT.

Components of NFY protein complexes that interact with the CCAAT and repressor element in the first exon are similar

The NFY transcription factor generally consists of 3 subunits: NFY-A, NFY-B, and NFY-C, which are all necessary for protein-DNA interaction.³² However, various subunits of NFY can dimerize and interact with other proteins.³³ We have demonstrated that NFY functions as both an activator and repressor of VWF promoter activity.

To determine whether the constituents of the NFY complexes that bind to the repressor element (at position +226 to +234) differ from those which bind to the CCAAT element (at position -18 to -14), we carried out supershift gel mobility assays with antibodies that specifically recognize the NFY-A, NFY-B, and NFY-C subunits. For these analyses, oligonucleotides corresponding to sequences -30 to -1 (which were centered around the CCAAT element, IR) and sequences +215 to +247 (the downstream NFY repressor binding site, R) were used as probes. Nuclear extracts from HeLa cells were incubated with either no antibodies, anti IgG, NFY-A, NFY-B, or NFY-C antibodies prior to adding the probes. The complexes were analyzed on a 5% nondenaturing acrylamide gel as described in Figure 3. The results demonstrate that the NFY complexes formed with both oligonucleotide probes are supershifted similarly by the anti-NFY-A and anti-NFY-B antibodies (Figure 8). Under these conditions we did not observe a supershift with the anti-NFY-C antibody in either case. However, if both the A and B subunits are present in these complexes it is most likely

that the C subunit is also present, but the protein-antibody interaction conditions may not be optimal for supershift using this anti-NFY-C antibody. In any case, these results demonstrate that the constituents of the NFY complexes that interact with the repressor and CCAAT element are similar.

Efficiency of NFY interaction with the CCAAT element is higher than that of the repressor element

Our data demonstrate that the NFY complex that interacts with the repressor and the CCAAT sequences contains similar components. However, the binding affinity of NFY for these 2 distinct sequences could be different, and thus contribute toward a mechanism for NFY's opposing roles as activator and repressor. To address this possibility, we carried out gel mobility experiments with the R- and CCAAT-containing IR probes in the presence of specific NFY competitors. The dissociation of the specific NFY-DNA complexes was determined by analyzing the complexes at various time points after addition of a fixed amount of the NFY-binding oligonucleotide competitor, and in an independent experiment in the presence of various concentrations of the competitor.

The time-dependent analysis demonstrates that the dissociation of the NFY complex from the oligonucleotide probe corresponding to the R element occurs almost immediately in the presence of the NFY competitor. However, dissociation of the NFY complex from the CCAAT-containing oligonucleotide probe takes approximately 30 minutes after addition of the competitor (Figure 9B).

The concentration-dependent analysis demonstrates that a 1- to 5-fold excess of the NFY-specific competitor was sufficient to reduce NFY complex formation with the repressor element by 60%, whereas a 10-fold excess of the same competitor was

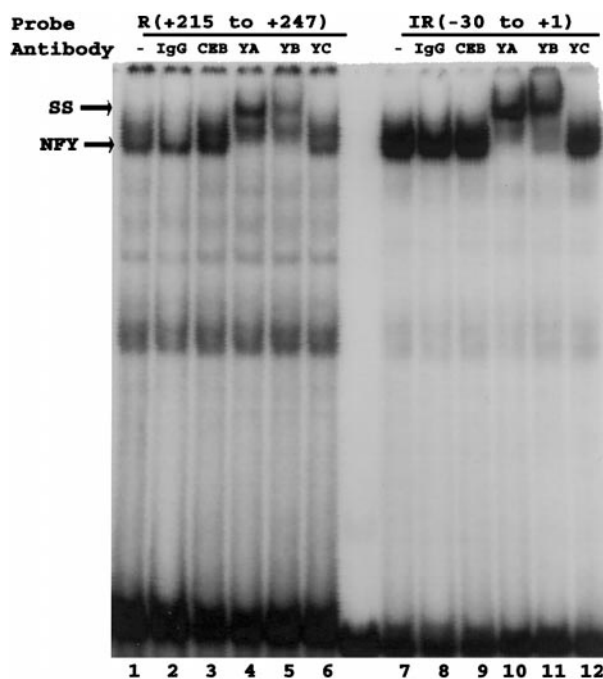


Figure 8. Determination of the subunit composition of NFY complexes. Nuclear extracts from HUVECs (5 μ g) were incubated with IgG and anti-C/EBP (shown as CEB) antibodies as negative controls, and anti-NFY-A, anti-NFY-B, and anti-NFY-C antibodies (1 μ g each). Following 3 hours of incubation at 4°C, the reaction mixtures were further incubated at room temperature for 15 minutes in the presence of either the IR probe that corresponds to sequences -30 to +1 and contains the CCAAT element (lanes 7-12), or the R probe corresponding to sequences +215 to +247 (lanes 1-6). Complexes formed were analyzed on an acrylamide gel as described for Figure 2.

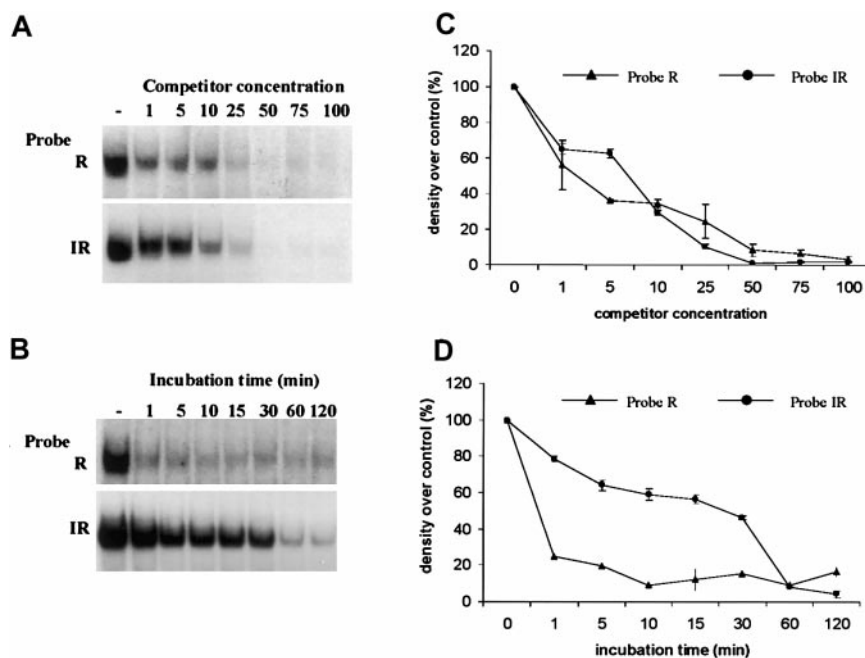


Figure 9. Determination of comparative dissociation rate of NFY when interacting with consensus and nonconsensus binding sequences. HeLa cell nuclear extracts (8 μ g) and oligonucleotide probes R and IR (corresponding to sequences +215 to +247 and -30 to +1, respectively) were used in gel mobility experiments as previously described. (A) For the concentration-dependent dissociation rate experiment, DNA probes and nuclear extracts were incubated for 20 minutes in the presence of various concentrations of double-stranded NFY competitor (ranging from 1 \times -100 \times excess of probe). (B) For the time-dependent dissociation rate experiment, the oligonucleotide probes were incubated independently with nuclear extract for 10 minutes prior to the addition of a 50-fold excess of double-stranded NFY competitor. Reaction mixtures were further incubated at 4 degrees for various time points (minutes) as shown above each lane. Complexes formed in both time- and concentration-dependent experiments (A and B) were analyzed on 5% nondenaturing acrylamide gel as described in Figure 2. Lane “-” in both A and B represents the DNA-NFY protein complex formed in the absence of competitor. (C, D) Densities of protein-DNA complexes were quantitated by phosphorimager analysis and the value obtained for protein-DNA complexes in the absence of competitors were used as control references. Densities of all other protein-DNA complexes in the presence of competitors were determined as percentages of control references for each experiment. The graphs represent the average of 2 independent analyses for each autoradiograph and the error bars represent the standard deviations. The solid circle represents the values obtained for the protein complex with the IR (-30 to +1) probe, and the solid triangle represents the values obtained for the protein complex with the R (+215 to +247) probe.

required to achieve the same level of inhibition of complex formation with the CCAAT-containing probe (Figure 9A).

These results demonstrated that the binding efficiency of NFY for the CCAAT sequence is significantly greater than that of the repressor sequence.

Discussion

The activation of the VWF gene promoter in endothelial cells is regulated by a complex mechanism that involves a number of activators and repressors. The role of repressors is important specifically in the maintenance of an inactivated state of the promoter in nonendothelial cells.¹⁹ We had previously demonstrated that a protein complex, designated “R,” interacts with sequences +215 to +247 of the VWF promoter and inhibits promoter activation in smooth muscle cells.²³ Using competition and supershift gel mobility assays, we now identify this “R” protein complex as the NFY factor. Transfection analysis in HEK 293 cells (as well as our previous transfection in BSM cells) confirmed the role of this NFY-DNA interaction as a repressor of VWF promoter activity in nonendothelial cells.

The observation that the NFY-DNA complex is present in both endothelial and nonendothelial cells is consistent with the presence of other repressors (NF1 and Oct1) of the VWF promoter in both cell types. We could not demonstrate the repressor function of the NFY in endothelial cells since the promoter sequences that contain this repressor NFY binding site are already active in endothelial cells; thus, the gain of activity that is the assay for the role of

repressor could not be determined. Based on these observations, we hypothesize that repressors have inhibitory functions on the VWF promoter in all cell types, however, an endothelial cell-specific mechanism exists that overcomes this inhibitory function specifically in endothelial cells. Such a mechanism may involve the presence of endothelial-specific activators that directly interact with DNA or coactivators that may interact and modify the function of either activators or repressors.

The sequence analysis of the +215 to +247 region did not reveal the presence of the CCAAT sequence that has been reported as an absolute requirement for NFY interaction with DNA. DNA methylation interference, mutation, and competition gel mobility assays demonstrated that the sequence CCGNNNCCC (+226 to +234) in the VWF promoter constitutes the binding site of NFY. Recently, another novel binding site for NFY in the promoter of the CHOP gene was also reported.³⁴ The NFY binding site in the CHOP promoter constitutes a CGTGC sequence as well as a CCAAT sequence, and NFY was shown to interact with both elements.³⁴ Interaction of NFY with the commonly identified CCAAT element is generally known to be necessary for basal constitutive promoter activation, although NFY also mediates cell-specific and inducible gene expression.^{33,35-37} Our results demonstrate that NFY can also interact with nonconsensus sequences that do not include the CCAAT element, and it can function as a transcription repressor as well as an activator.

The VWF promoter also contains a consensus CCAAT element spanning nucleotides -18 to -14 in its 5' region. We have recently demonstrated that NFY also interacts with this CCAAT element and through this interaction mediates the irradiation induction of

the core (sequences -90 to +22) VWF promoter that is not endothelial specific and active in all cell types.³¹ Here we have further analyzed the role of NFY when it interacts with the CCAAT element in the VWF promoter. Our results demonstrate that in the context of the sequences (-487 to +247) that constitute the endothelial-specific VWF promoter fragment, NFY interaction with the CCAAT sequence is necessary for constitutive promoter activation in endothelial cells. These results demonstrated that the NFY transcription factor functioned as both an activator and a repressor of VWF promoter activity.

The NFY transcription factor is a heteromeric protein complex that consists of 3 subunits, NFY-A, NFY-B, and NFY-C.³² Alternative splicing generates different isoforms of the A subunit in some cell types, and subunits of NFY can interact with other proteins, thus providing a mechanism for the formation of NFY complexes with variable components.^{36,33} We have recently demonstrated that there is only a single NFY-A subunit in endothelial cells.³¹ Here we have also analyzed the components of NFY that interact with the CCAAT and the repressor element and show that the constituents of the NFY complex that bind to these sites are similar. However, we have also shown that NFY has a significantly greater binding affinity for the consensus CCAAT sequence compared to the nonconsensus repressor element. Our results suggest that neither different A subunit isoforms nor the basic composition of NFY

appear to be the mechanism by which NFY performs its dual function in regulation of VWF gene expression.

Other transcription factors also have dual functions. The transcription factor YY1 can function as either an activator or a repressor depending on its binding sequence and/or its interaction with specific cofactors.^{38,39} Some transacting factors, including NFY, interact with coactivators such as p300/CBP and PCAF that function as histone acetylases, and others can interact with corepressors that function as histone deacetylases.⁴⁰⁻⁴⁴ A switch between the activator and repressor functions of YY1 is correlated to interactions with factors that function as coactivators or corepressors.^{45,46}

Based on these results, we hypothesize that NFY can play different roles in the regulation of gene activation, and these roles may be partially dependent on the NFY binding sequence. Although the basic components of the NFY complexes that interact with the VWF promoter are similar, we hypothesize that the dual function of NFY may be regulated through recruitment of different cofactors.

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