

Cell type–specific regulation of von Willebrand factor expression by the *E4BP4* transcriptional repressor

Christine Hough, Carla D. Cuthbert, Colleen Notley, Christine Brown, Carol Hegadorn, Ergul Berber, and David Lillicrap

Mechanisms of tissue-restricted patterns of von Willebrand factor (*VWF*) expression involve activators and repressors that limit expression to endothelial cells and megakaryocytes. The relative transcriptional activity of the proximal *VWF* promoter was assessed in *VWF*-producing and -nonproducing cells, and promoter activity was highest in endothelial cells followed by megakaryocytes. Only basal *VWF* promoter activity was seen in nonendothelial cells. Here we identify a negative response element located at nucleotides (nts) +96/+105 and demon-

strate, using chromatin immunoprecipitation (ChIP) analysis, that in vivo this sequence interacts with the *E4BP4* transcriptional repressor. Differences in size and relative abundance of nuclear *E4BP4* were observed. In HepG2 cells, low levels of larger forms of *E4BP4* are present that directly interact with the negative response element. In *VWF*-expressing cells, high levels of smaller forms predominate with no evidence of direct DNA binding. However, in endothelial cells, mutation of the *VWF* *E4BP4* binding motif not only restores but also further elevates *VWF*

promoter activity, suggesting that *E4BP4* may be part of a coordinated binding complex. These observations implicate this binding motif in repressing both activated and basal levels of *VWF* transcription by different cell type–specific mechanisms, and support the hypothesis that *E4BP4* sequesters negative regulators of transcription, thereby enhancing activated gene expression. (*Blood*. 2005;105:1531-1539)

© 2005 by The American Society of Hematology

Introduction

Von Willebrand factor (*VWF*) is synthesized exclusively in endothelial cells and megakaryocytes.¹⁻³ It mediates the interaction between platelets and components of the subendothelium at sites of vascular injury, and in the circulation protects the coagulation factor VIII from proteolytic degradation.⁴⁻⁷ The critical role that *VWF* plays in normal hemostasis is illustrated by the effects of abnormally low or high plasma *VWF* levels. Deficiencies of *VWF* result in the bleeding diathesis von Willebrand disease, while elevated levels are associated with pathophysiologic processes such as coronary artery thrombosis.⁸ There is considerable variation in plasma *VWF* levels between individuals,⁹ but as well, within an individual there is a marked regional heterogeneity in *VWF* expression in endothelial cells throughout the vasculature.¹⁰⁻¹² Significant efforts have been directed at understanding transcriptional regulatory mechanisms that control not only cell lineage–specific expression but also the observed variations in *VWF* levels.^{11,13,14}

Initial characterization of the 5′-flanking region of the *VWF* promoter identified the presence of positive and negative regulatory elements as far upstream as 2 kilobases (kb) from a single transcriptional start site.¹⁵ The structural organization and nucleotide sequence of the human, bovine, and murine proximal *VWF* promoters, including the first exon, are quite similar.^{16,17} In vitro analysis of the human *VWF* promoter identified a minimal core promoter between nucleotides (nts) −90 and +22 that is capable of

inducing transcription in both endothelial and nonendothelial cells.¹⁸ In vitro, the region that spans nts −487 to +247 imparts endothelial-specific promoter activity¹⁸ but is repressed in all cell types by an upstream element located at nts −487/−440 that interacts with an NF-1–like transcriptional repressor.^{19,20} Enhanced transcription of the *VWF* gene is controlled in part by the positive regulatory region (+145 to +247) of the promoter that also contains *GATA* consensus binding sites at nt +220 and nt −80. These elements relieve the transcriptional inhibition of the upstream negative regulatory element in an endothelial cell–specific manner.^{20,21} A putative *GATA* binding element has also been identified at nt +53 of the *VWF* promoter.²² Members of the Ets family of transcription factors bind to the consensus site located at nt −56 and also up-regulate *VWF* promoter activity in both endothelial cells and HeLa cells.²¹ An additional negative regulatory element has been localized between nts −133 and −125 that binds the Oct-1 transcription factor.²³ Finally, the *NFY* transcription factor serves either as an activator or a repressor of *VWF* promoter activity.^{19,24} *NFY* acts as an activator when it binds to its consensus sequence at nt −18 of the promoter, but functions as a repressor in nonendothelial cells through the recruitment of histone deacetylase to sequences +226 to +234.²⁵ This region of the promoter also interacts with *GATA6*, which *trans*-activates the *VWF* promoter in an endothelial-specific manner.²⁵

From The Department of Pathology and Molecular Medicine, Richardson Laboratories, Queen's University, Kingston, ON.

Submitted October 15, 2002; accepted October 18, 2004. Prepublished online as *Blood* First Edition Paper, October 21, 2004; DOI 10.1182/blood-2002-10-3093.

Supported by operating grants from the Heart and Stroke Foundation of Ontario (T4421) and the Canadian Institutes of Health Research (MOP42467). D. L. is the recipient of a Career Investigator Award from the Heart and Stroke Foundation of Ontario and a Canada Research Chair in Molecular Hemostasis.

C.H. and C.D.C. contributed equally to this work.

Reprints: David Lillicrap, The Department of Pathology and Molecular Medicine, Richardson Laboratories, Queen's University, Kingston, ON, Canada, K7L 3N6; e-mail: lillicrap@cliff.path.queensu.ca.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 U.S.C. section 1734.

© 2005 by The American Society of Hematology

Transgenic mouse models have been used to elucidate the underlying mechanism that confers endothelial-specific expression of VWF in vivo. Surprisingly, the region spanning nts -487 and $+246$ that limits expression to endothelial cells in vitro, directs expression only in blood vessels of the brain.¹¹ Even the inclusion of an additional 2 kb of 5' flanking sequences confers expression only to the endothelium of the brain, heart, and skeletal muscle,²⁶ suggesting that additional regions of the *VWF* locus are required to direct ubiquitous endothelial expression of VWF.

Clearly, the molecular mechanisms involved in regulating VWF expression in endothelial cells are complex. It has been proposed that VWF is regulated in a modular fashion in so far as its expression is mediated by the overall interaction of distinct signaling pathways that interact with different regions of the *VWF* promoter.^{10,26} With a view to better understand the cell lineage-specific nature of VWF expression, we studied the proximal promoter region of the *VWF* gene in bovine aortic endothelial cells (BAECs), differentiated and undifferentiated megakaryocytic cells (Dami), and a non-VWF-expressing cell line, HepG2. Here we describe the presence of a transcriptional repressor element located between nts $+96$ and $+105$ and show that this *cis*-acting element interacts with the adenovirus E4 promoter-binding protein 4 (E4BP4) transcription repressor to regulate *VWF* promoter activity.

Materials and methods

Cell culture

The human megakaryoblastic cell line Dami, hepatoma cell line HepG2, baby hamster kidney cell line, BHK, and the human epitheloid carcinoma cell line, HeLa, were all obtained from the American Type Culture Collection (ATCC, Rockville, MD). Human umbilical vein endothelial cells (HUVECs) were obtained from Clonetics (Walkersville, MD). HepG2, HUVEC, and HeLa cell lines were grown in minimal essential media (MEM); BHK cells were grown in Dulbecco modified Eagle medium (DMEM); and all cultures were supplemented with 10% heat-inactivated fetal bovine serum. Both undifferentiated Dami and differentiated Dami cells were maintained in Iscoves modified Dulbecco medium (IMDM) supplemented with 10% heat-inactivated horse serum. To induce undifferentiated Dami cells to differentiate, 5×10^5 to 2×10^6 cells/mL were cultured for 3 to 5 days in media that contained either 1.25% dimethyl sulfoxide (DMSO) or 50 nM phorbol myristate acetate (PMA) with 0.25% DMSO.²⁷ Primary BAECs were isolated using the method of Gimbrone,²⁸ or were obtained from Clonetics. Cell passages between 4 and 8 were maintained in DMEM. All cell culture media contained 100 U/mL penicillin and 100 μ g/mL streptomycin and were incubated at 37°C in 5% CO₂.

Generation of reporter constructs

Using human *VWF* gene sequence as the template, polymerase chain reaction (PCR) products were generated with the upstream and downstream primers as indicated in Table 1 and cloned into the TA vector pCR2.1 (Invitrogen, Carlsbad, CA). Fragments were sequenced, digested, and subcloned into pGL2-Basic or pGL2-Promoter (Promega, Madison, WI). To generate constructs that contained a single copy of the consensus E4BP4, *VWF* E4BP4, or the mutated *VWF* E4BP4 binding sites, oligonucleotides listed in Table 2 were annealed and ligated into the linearized pGL2-Promoter reporter vector.

Generation of the mutated E4BP4 binding site

The TAA (nts $+102$ to $+104$) of the E4BP4 binding site in the *VWF* promoter was converted to CTG by site-directed mutagenesis. A 340-bp region of the *VWF* promoter between the *Xho*I and *Hind*III was subcloned into the pBK-CMV vector (Stratagene, La Jolla, CA). Site-directed

Table 1. Primer sets used to create *VWF* promoter deletion constructs

PGL2 basic constructs (S/AS)	Primer sequence (5'-3')
VWF-Prom (-91 -S)	AAAGCTTTATCAGCTTTGGAG
VWF-Prom ($+229$ -AS)	GCGGTATCTCCAAGGTCCT
VWF-Prom (-63 -S)	AATACCATTTCCTTTCATTGTT
VWF-Prom ($+247$ -AS)	CCCTGCAAAATGAGGGTGC
VWF-Prom (-63 -S)	AATACCATTTCCTTTCATTGTT
VWF-Prom ($+169$ -AS)	GGCCATGCTCAGCTGCTGC
VWF-Prom (-63 -S)	AATACCATTTCCTTTCATTGTT
VWF-Prom ($+70$ -AS)	GGGGAGATAAAGCCCAAGCT
VWF-Prom ($+53$ -S)	TTGGGCTTTATCTCCCCAGC
VWF-Prom ($+229$ -AS)	GCGGTATCTCCAAGGTCCT
VWF-Prom ($+53$ -S)	TTGGGCTTTATCTCCCCAGC
VWF-Prom ($+169$ -AS)	GGCCATGCTCAGCTGCTGC
VWF-Prom ($+53$ -S)	TTGGGCTTTATCTCCCCAGC
VWF-Prom ($+145$ -AS)	GGCTCAATCAGGCTGCATC
VWF-Prom ($+170$ -S)	AGAGCATGGCCTAGGGTGGG
VWF-Prom ($+229$ -AS)	GCGGTATCTCCAAGGTCCT
VWF-Prom ($+63$ -S)	TCTCCCCAGCAGTGGGGACT
VWF-Prom ($+102$ -AS)	TGTAGCCAGGGGCTGTGGA
VWF-Prom ($+83$ -S)	TCCACAGCCCTGGGCTAC
VWF-Prom ($+121$ -AS)	CCGGACTGCTCTGCTGTTATG

S indicates sense; AS, antisense.

mutagenesis was performed using the Quikchange Multi-Site Directed Mutagenesis Kit (Stratagene) according to the manufacturer's protocol.

Cell transfection and luciferase assay

Large-scale DNA preparations of VWF reporter constructs were purified using Qiagen columns (Qiagen, Valencia, CA) according to the manufacturer's instructions. Dami cells were transfected using Effectene Reagent (Qiagen) with 550 ng of the VWF reporter construct and 50 ng of an expression vector for β -galactosidase (pCMV- β GAL). Transfections of BAECs and BHK, HeLa, and HepG2 cells were carried out by the calcium phosphate coprecipitation method²⁹ in 35-mm² plates using 2.5 μ g of the VWF reporter constructs and 0.5 μ g of pCMV- β GAL. Luciferase activity (Promega Luciferase Assay) and β -galactosidase activity (Tropix Galacto-Light System; Applied Biosystems, Bedford, MA) were measured according to the manufacturer's instructions using a Berthold Lumat LB9501 luminometer (Berthold Technologies, Oak Ridge, TN). Luciferase activity was normalized with respect to β -galactosidase activity to correct for differences in transfection efficiencies and cell numbers. The E4BP4 expression construct was a kind gift from Dr Helen Hurst (Hammersmith Hospital, London, United Kingdom).

Gel mobility shift assays

Double-stranded oligonucleotides were designed for the E4BP4 consensus-binding site, the *VWF* E4BP4 binding region (Neg 5), and the mutated *VWF* E4BP4 binding region (Neg 5 Mut) (Table 3) and labeled with 15 μ Ci (0.555 MBq) [α -³²P] deoxyadenosine triphosphate (dATP). Nuclear protein extracts from BAECs, HUVECs, and HepG2 cells were prepared using the method of Dignam³⁰ and from Dami cells by the method of Sierra.³¹ Extracts (5-10 μ g) were incubated at room temperature for 20 minutes in binding buffer (25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], pH 7.6; 5 mM MgCl₂, 34 mM KCl, 0.05 μ g/ μ L poly poly(deoxyinosinic-deoxycytidylic) acid sodium salt [dI:dC], 1 μ g/ μ L bovine serum albumin) and 100 000 cpm ³²P-labeled probe. In competition studies, a 100-fold molar excess of unlabeled double-stranded oligonucleotide was also included in the binding reaction. For the antibody supershift assays, nuclear protein was incubated at room temperature for 15 minutes with 4 μ g of an antihuman E4BP4 antibody (no. SC9549X; Santa Cruz Biotechnology, Santa Cruz, CA) after the initial incubation with the labeled oligonucleotide probe. Preimmune serum was used as a control for nonspecific binding of the antibody.

Table 2. Complementary oligonucleotides used to create single-site reporter constructs

PGL2 promoter constructs	Annealed oligonucleotides
VWF E4BP4 (Neg 5)	5' GATC GGGCTACATAAC CCCAGTGTATTG GATC 5'
Mutant VWF E4BP4 (Neg 5 Mut)	5' GATC GGGCTACACTGCAGCA CCCAGTGTGACGTCGT GATC 5'
E4BP4 consensus	5' GATC GTTATGTAACG CAATACATTGC GATC 5'

Western blot analysis

Nuclear protein samples were diluted into loading buffer that contained reducing agent and separated on a sodium dodecyl sulfate (SDS)–polyacrylamide gel (7.5%) in Tris (tris(hydroxymethyl)aminomethane)–glycine buffer. Molecular mass markers were purchased from New England Biological (catalog no. 7707S; Beverly, MA) and Bio-Rad (catalog no. 161-0374; Mississauga, ON). After electrotransfer, immunoblotting was performed with a polyclonal anti-E4BP4 antibody (no. SC9550; Santa Cruz Biotechnology). Preincubating the antibody with its blocking peptide prior to immunoblotting was carried out to assess the specificity of the E4BP4 antibody. Blots were incubated with affinity-purified anti-rabbit immunoglobulin G (IgG) horseradish peroxidase–conjugated secondary antibody (Affinity Biological, Hamilton, ON).

Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was carried out using the ChIP Assay Kit from Upstate Biotechnology (Lake Placid, NY) as recommended by the manufacturer. Total chromatin was removed prior to immunoprecipitation to serve as an input sample. Immunoprecipitation using anti-E4BP4 antibodies (nos. SC9550 and SC9549X; Santa Cruz Biotechnology) was performed at 4°C for 16 hours. A negative control without antibody was also carried out. Human VWF and β-actin promoter sequences were amplified using primer pairs and conditions outlined in Table 4.

Amplification of the E4BP4 RNA

Total RNA was isolated from the various cell types using TRIzol Reagent (Life Technologies, Bethesda, MD) according to the manufacturer’s instructions. Reverse transcription and PCR amplification were carried out using the primers and conditions outlined in Table 4.

Results

Relative transcriptional activity of the VWF proximal promoter in VWF-expressing and non-VWF-expressing cells

To characterize the VWF promoter region that encompasses the 2 GATA elements at nts –80 and +220, and to identify elements that influence transcription, various deletion constructs spanning this region were engineered (Figure 1A). To assess the cell type–specific relative transcriptional activity of the proximal promoter, the region spanning nts –91 to +229 was transfected into VWF-expressing and -nonexpressing cells. (Figure 1B). This region of the promoter is in the order of 40-fold more transcriptionally active in endothelial cells (BAECs) compared with nonendothe-

Table 3. Sequence information of the oligonucleotides used in electrophoretic mobility shift assays

Site	Sequence (5'-3')
E4-BP4 consensus	GGTGATCCGTTTATGTAACGGATCC
NEG 5	GGTGCTGGGCTACATAACAGATCC
	← ←E4BP4 site
NEG 5 Mut	GGTGCTGGGCTACACTGCAGATCC

lial cells (HepG2, BHK, and HeLa), and in undifferentiated megakaryocytes it is about 4-fold less than that seen in the endothelial cells. However, once Dami cells are induced to differentiate, the promoter activity more than doubles.

Identification of repressor activity in the first noncoding exon of the VWF gene

The VWF promoter activity of the various deletion constructs, relative to the construct containing nts –91 to +229, was assessed in endothelial cells, undifferentiated and differentiated megakaryocytes, and non-VWF-expressing HepG2 cells (Figure 1C). Deletion of the GATA element located at nt –80 resulted in a loss of transcriptional activity only in VWF-producing cells. In HepG2 cells, deletion of this element resulted in an increase in transcriptional activity. Deletion of most of the positive regulatory region (+169 to +247) resulted in a significant loss of promoter activity in all cell types analyzed. Additional deletion of DNA sequences between nts +70 and +169 resulted in a significant increase in promoter activity only in the cells that synthesize VWF, suggesting the presence of a cell type–specific repressive element. To localize this possible repressive element, deletion constructs spanning nts +53 to +229 (Figure 2A), cloned upstream of the simian virus 40 (SV40) promoter and the luciferase reporter gene, were transfected into VWF-producing and non-VWF-producing cells (Figure 2B). The results indicate that these VWF promoter sequences are capable of repressing heterologous SV40 promoter activity by 4-fold in both endothelial cells and differentiated megakaryocytes. There was also a reduction in SV40 promoter activity, albeit less pronounced, when these constructs were transfected into HepG2 cells. These results, in conjunction with the results presented in Figure 1C, localize the repressive element to a region between nts +70 to +145. When the DNA fragment containing sequences from nts +170 to +229 was transfected into the 3 cell types, a restoration of promoter activity was seen in the HepG2 cells, while a doubling in promoter activity, compared with baseline values, was seen in both the differentiated megakaryocytes and endothelial cells, as would be expected from increased transactivation mediated by the GATA element at nt +220.

Table 4. Sequence information of the oligonucleotides used for PCR amplification

Primer	Sequence (5'-3')	PCR condition
E4BP4-nt-2-S	CCCTTTCTTTCTCCTCGTC	Hot start
E4BP4-nt-1521-AS	CCCCTGCTTCAAATACAAGTT	94°C 4 min
E4BP4-nt-562-S	GCTGAGCTGCTTTCACATAA	94°C 45 s
		52°C 45 s
		72°C 2 min
		35 cycles
VWF-nt-3-S	CACAGCTATTGTGGTGGGAAA	Hot start
VWF-nt-225-AS	CAAATGAGGGCTCGCGCTATC	94°C 4 min
		94°C 45 s
		60°C 45 s
		72°C 45 s
		30 cycles
β-actin1-S	TGCCTAGGTCACCCACTAATG	Hot start
β-actin2-AS	GTGGCCCGTGATGAAGGCTA	94°C 4 min
		94°C 45 s
		60°C 45 s
		72°C 45 s
		30 cycles

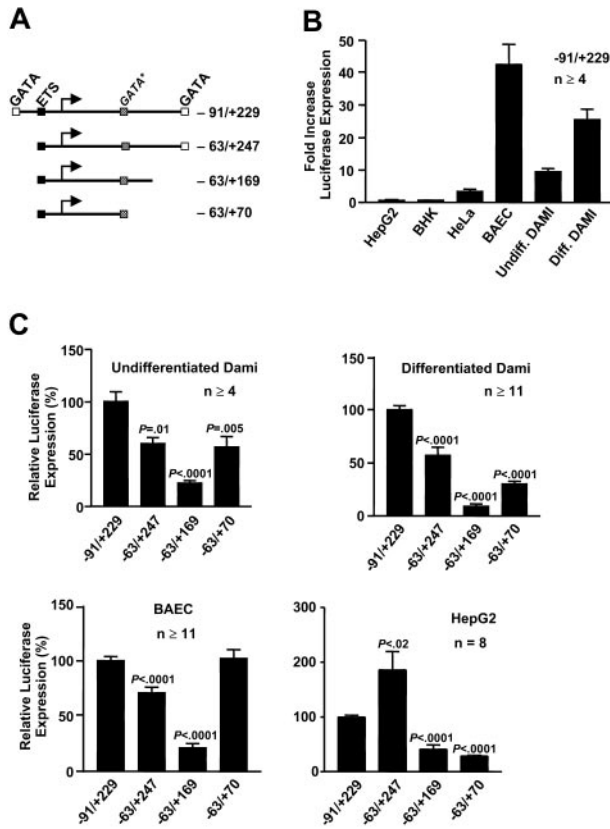


Figure 1. Deletion analysis and relative expression of the human VWF proximal promoter region in VWF-expressing and non-VWF-expressing cells. (A) Diagrammatic representation of the proximal VWF promoter including the first noncoding exon cloned upstream of the luciferase gene. Nucleotide sequence numbers are indicated with respect to the human VWF transcription start site. Open boxes represent GATA sites at nts -81 and +220. A GATA element (□) located at nt +53 appears to enhance transcription in megakaryocytes.²² The Ets element (■) and the transcriptional start site (arrow) are indicated. (B) The mean fold increase in normalized luciferase expression of the promoter construct containing nts -91 to +229, relative to the promoterless pGL2 Basic construct, transfected into VWF-producing and -nonproducing cells. (C) Mean normalized luciferase expression relative to the full-length VWF promoter (nts -91/+229) is expressed as a percentage. n represents the number of experiments for each of the cell types. Error bars indicate the standard error of the mean. Results of *t* tests between specific deletion constructs and the full-length proximal promoter are shown, and a *P* value is considered statistically significant when *P* < .05.

Localization of a putative repressor element to nucleotides +96/+105

To further delineate the location of the repressor activity, 2 reporter constructs were generated that encompass nts +63 to +121, cloned upstream of the SV40 promoter. These constructs were compared for transcriptional activation in VWF-producing and -nonproducing cells (Figure 3A). In VWF-producing cells, the construct containing nts +83 to +121 of the VWF promoter exhibited a 3.5- and 2.3-fold reduction in SV40 promoter activity in BAEC and Dami cells, respectively, while only a 1.5-fold reduction in activity was observed in HepG2 cells. The construct containing +63 to +102 showed increased repression when compared with +83 to +102 in HepG2 cells, however the difference was not statistically significant.

The nucleotide sequence spanning +83 to +121 was subjected to a transcription factor binding site homology search using the database located at <http://www.cbrc.jp/research/db/TFSEARCH.html>, and a putative element between nts +96/+105 (in the reverse orientation) was identified that bore close

homology to an E4BP4 consensus binding site (Figure 3B) with a one-base pair mismatch of the 10-base pair consensus sequence. E4BP4 was first characterized by Cowell and Hurst³² and Cowell³³ as a ubiquitously expressed transcriptional repressor, and a novel member of the basic/leucine zipper (b-ZIP) family of transcription factors.

The E4BP4 transcriptional repressor binds to the *cis*-element at nucleotides +96/+105 in HepG2 cells and represses basal VWF promoter activity

To investigate the protein-binding properties of the putative repressor element, a series of double-stranded oligonucleotides were generated (Table 3). The Neg 5 oligonucleotide contains the putative VWF E4BP4 element, while Neg 5 Mut contains the mutated putative VWF E4BP4 element (nts +102 to +104 mutated from TAA to CTG). Electrophoretic mobility shift assays (EMSA) and supershift assays were performed with nuclear proteins derived from HepG2 cells transfected with an E4BP4 expression plasmid, and untransfected HepG2 cells, and the results are presented in Figure 4A and 4B, respectively. The transfected HepG2 cells were used as a positive control for E4BP4 nuclear protein. Labeled oligonucleotides corresponding to the E4BP4 binding site consensus and the putative VWF E4BP4 site show a similar smear of gel-retarded complexes. This smear is expected as bZIP transcription factors undergo extensive homo- and heterodimerization, and recognize similar DNA binding elements.^{34,35} Neg 5 Mut does not show a similar smear of gel-retarded DNA/protein complexes (Figure 4C). The supershift assays show slower migrating complexes with both the Neg 5 and E4BP4 oligonucleotides, which clearly indicate that the E4BP4 directly interacts with the VWF E4BP4 binding element. The presence of a very faint complex in the non-E4BP4-transfected HepG2 cells mirrors the basal level of the endogenous VWF promoter activity in these cells and shows that E4BP4 is a minor component of the bZIP proteins that interact with these DNA sequences. Similar results have been reported for the E4BP4 consensus sequence.³⁴ Replacement of the E4BP4 antibody with preimmune serum confirms that the supershifted

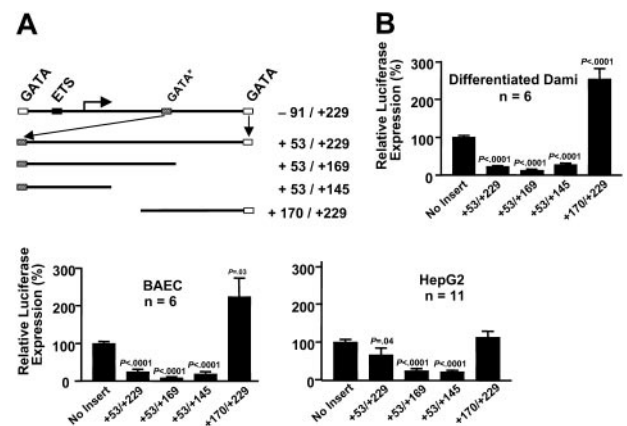
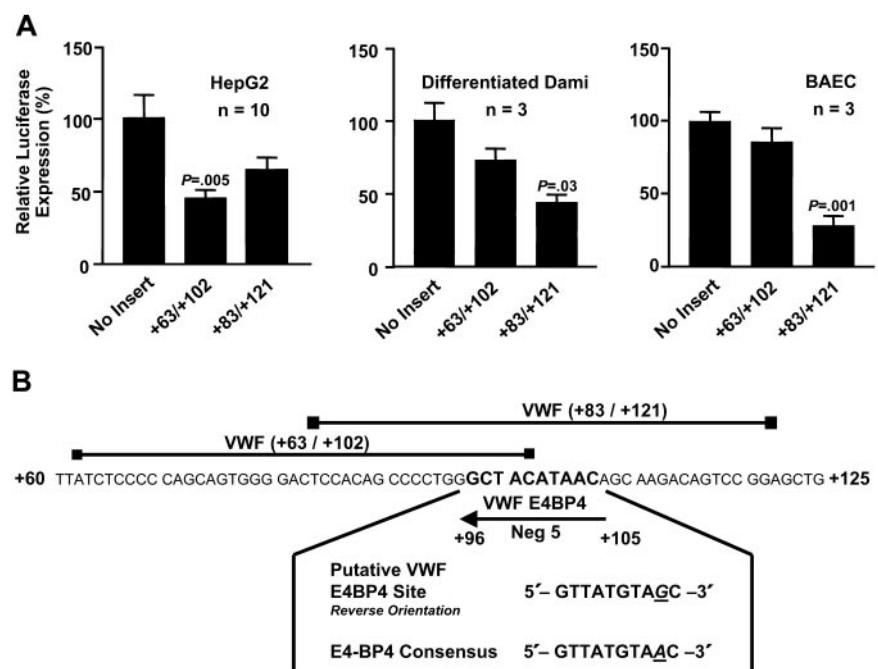


Figure 2. The proximal VWF promoter spanning nucleotides +53/+240 can repress the heterologous SV40 promoter in VWF-producing and non-VWF-producing cells. (A) Diagrammatic representation of the VWF promoter region cloned upstream of the SV40 promoter and luciferase reporter gene. Nucleotide sequences, GATA and Ets sites, and transcriptional start site are indicated as outlined in Figure 1. (B) The mean normalized luciferase expression relative to a "No insert" control pGL2 construct is graphed as a percentage. The number (n) of experiments carried out is indicated on each graph. The error bars indicate the standard error of the mean. Statistical differences between each deletion construct and the pGL2 control construct were assessed using a Student *t* test, and a *P* value of less than .05 is considered significant.

Figure 3. Localization of the negative response element. (A) The 2 fragments spanning +63 to +121 of the first exon of the *VWF* gene cloned upstream of the SV40 promoter and luciferase reporter gene. Mean normalized luciferase expression relative to a “No insert” control pGL2 construct is graphed as a percentage. The number (n) of experiments carried out is indicated on each graph. The error bars indicate the standard error of the mean. Statistical differences between each deletion construct and the pGL2 control construct were assessed using a Student *t* test, and a *P* value of less than .05 is considered significant. (B) A diagrammatic representation of the nucleotide sequences included in the constructs tested in panel A. The *VWF* E4BP4 binding element is shown.



DNA/protein complex is not a nonspecific complex (Figure 4D). Competition binding assays using a 100-fold molar excess of unlabeled E4BP4, Neg 5, and Neg 5 Mut as a competitor for binding were carried out with nuclear proteins isolated from HepG2 cells (Figure 4E). When Neg 5 was used as the labeled probe, both E4BP4 and Neg 5, but not Neg 5 Mut, were able to efficiently compete away the gel-retarded DNA/protein complexes. Similar results were seen when the E4BP4 consensus sequence was used as the labeled probe.

To confirm that the *VWF* E4BP4 element is able to function as a *cis*-acting element through which the E4BP4 transcriptional repressor can mediate inhibition of transcription, several reporter constructs were generated for transfection into HepG2 cells. Constructs contain one copy of either the *VWF* E4BP4 element, the mutated *VWF* E4BP4 element, the E4BP4 consensus element, nts +63/102 of the *VWF* promoter, or nt +83/+121 of the *VWF* promoter, cloned upstream of the luciferase gene, regulated by the SV40 promoter. These constructs were cotransfected into HepG2 cells with increasing concentrations of an E4BP4 expression vector. Designating the expression of the construct in the absence of the E4BP4 expression vector as one, the fold repression with increasing amounts of E4BP4 vector was determined for each reporter construct and is represented in Figure 4F. The control construct without any E4BP4 elements showed no increase in transcriptional repression, while the presence of one copy of the *VWF* E4BP4 element, nts +83/121 of the *VWF* promoter, or the E4BP4 consensus-binding site was sufficient to mediate, in the order of a 4-fold repression of transcriptional activity when the maximum amount (10 μ g) of E4BP4 expression vector was used. Furthermore, the extent of repression increased with increasing amounts of transfected E4BP4 construct. In contrast, neither the mutated *VWF* E4BP4 element nor the construct containing sequence spanning nts +63/+102 from the *VWF* promoter demonstrated significant transcriptional repression. These results demonstrate that E4BP4 binds to the sequences at +96/+105 and represses *VWF* promoter activity, and the magnitude of this repression appears to depend of the amount of E4BP4.

Analysis of DNA binding of E4BP4 to the *VWF* promoter negative response element in *VWF*-producing cells

The E4BP4 binding element in the *VWF* promoter mediates the strongest repressive effect in endothelial cells. In BAECs, removal of the element restores transcription (5-fold) to levels seen only with the full-length proximal *VWF* promoter, which, unlike the deleted construct, contains additional GATA elements at -81 and +220 (Figure 1C). In differentiated and undifferentiated megakaryocytes, removal of the negative element results in 3- and 2-fold increases, respectively, in promoter activity. It would appear, then, that the negative response element mediates a restraint on transcription in *VWF*-producing cells. To investigate the protein-binding properties of E4BP4 to the negative response element, standard EMSA analysis using nuclear proteins isolated from *VWF*-expressing cells (Dami and BAECs) and the oligonucleotides containing the consensus E4BP4 and *VWF* E4BP4 binding sites was carried out. The results presented in Figure 5A show a diffuse smear of protein/DNA complexes, but this was evident only when 10 μ g nuclear protein was used with a prolonged exposure of the autoradiograph. No supershifted complex could be detected when the E4BP4 antibody was used. Nuclear proteins isolated from HUVECs also showed only negligible DNA binding with no supershifted complex (data not shown).

Western blot analysis of nuclear proteins

To determine if E4BP4 is present in the nucleus of *VWF*-producing cells, a Western blot was prepared with nuclear proteins isolated from HepG2 cells, BAECs, HUVECs, and Dami cells, and the results are shown in Figure 5B. Preincubating the antibody with its blocking peptide assessed nonspecific binding of the E4BP4 antibody to the nuclear proteins. Nonspecific bands of 35 and 62 kDa are observed. In HepG2 nuclear extracts, low levels of each of 2 proteins of approximately 60 and 63 kDa are observed, and these sizes correspond to those previously reported for E4BP4 in HuH-7 cells.³⁴ In BAEC nuclear extracts, however, an intense band corresponding to a molecular mass of about 53 kDa is observed,

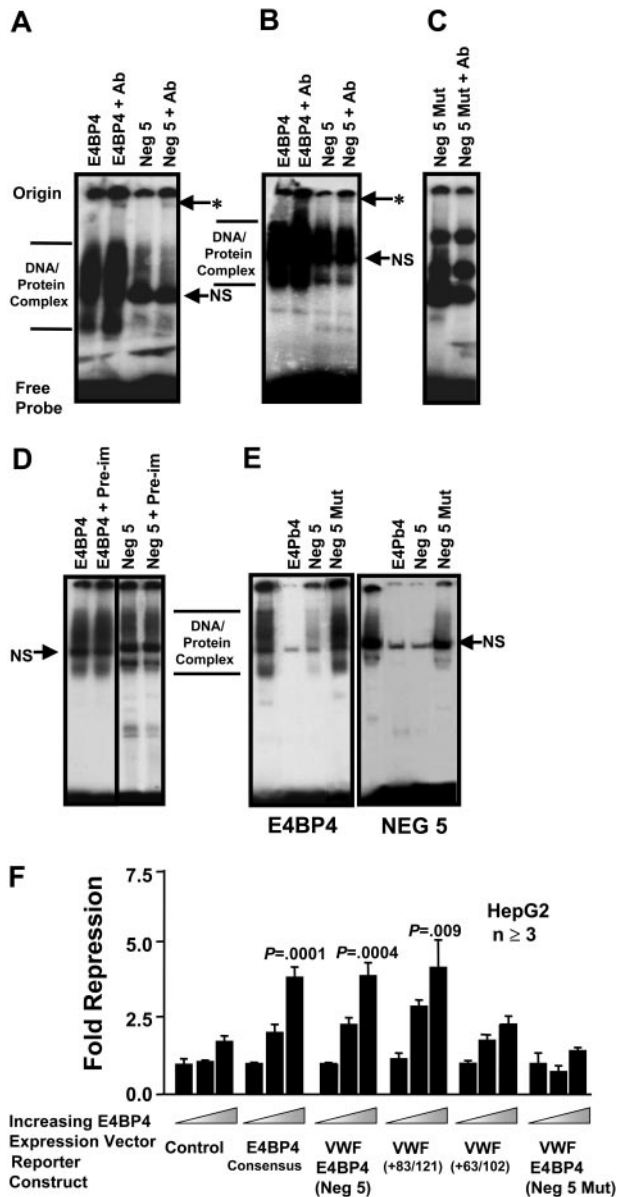


Figure 4. E4BP4 binds to the *VWF* promoter element and represses transcription in HepG2 cells. EMSA and supershift analysis of nuclear proteins isolated from (A) HepG2 cells transfected with the E4BP4 expression plasmid and (B-E) nuclear extracts from non-E4BP4-transfected HepG2 cells. Oligonucleotide probes contained the E4BP4 consensus binding element (E4BP4) or the *VWF* promoter E4BP4 binding element (Neg 5) or (C) a mutated *VWF* E4BP4 binding element (Neg 5 Mut). To supershift the DNA/protein complexes, an E4BP4 antibody (Ab) was used. An * identifies the very faint supershifted complex. (D) Specificity of the supershifted complex was confirmed by replacing the E4BP4 antibody with preimmune serum. (E) Competition binding assays using 32 P-labeled oligonucleotides for either E4BP4 or Neg 5 and competed away with unlabeled E4BP4, Neg5, or Neg 5 Mut oligonucleotides. NS indicates the presence of a nonspecific DNA/protein complex, and the free probe and well origin are shown. (F) HepG2 cells cotransfected with increasing concentrations of the E4BP4 expression vector and the various reporter constructs containing the indicated oligonucleotides upstream of the SV40 promoter. The mean normalized luciferase activity is expressed as fold repression relative to the reporter construct in the absence of the E4BP4 expression vector. Error bars represent standard error of the mean. A *t* test assessed for significant repression between cotransfection of the reporter construct in the absence or presence of the highest levels of the E4BP4 expression construct. *P* values are indicated above the constructs tested.

which was the reported mass when E4BP4 was first identified.³² In nuclear extracts isolated from undifferentiated and differentiated Dam1 cells, an intense band of approximately 33 kDa is observed, and this size of E4BP4 has never previously been reported. No

significant difference in the amount of E4BP4 is observed between the 2 cell types, indicating that it may not play a prominent role in modulating the levels of VWF expression when Dam1 cells are induced to differentiate. Apparently, only a very small amount of higher molecular weight E4BP4 is present in the nucleus of HUVECs. To determine if these forms of E4BP4 result from alternative splicing, and to evaluate the presence of the DNA binding domain, RNA was isolated from the various cell types and reverse transcribed and a fragment spanning nts 2 to 1521 was amplified (Figure 5C). The nucleotide sequence of the bovine *E4BP4* 5' untranslated region (UTR) is not known and presumably diverges from the human sequence as all attempts to amplify the *E4BP4* RNA from BAECs failed. A second fragment that spans nts 562 to 1521 of the only translated exon was therefore amplified from BAECs and compared with the identical fragment amplified from HUVECs. The 1.51-kb fragment isolated from HUVECs was sequenced and the predicted DNA binding motif was identified. The other amplified fragments were all assessed by restriction digest analysis. No evidence of alternative splicing was detected, suggesting that posttranslational modifications of E4BP4 (including possible proteolysis) must occur to account for the different E4BP4 forms seen in these studies.

Mutation of the *VWF* E4BP4 binding element restores *VWF* promoter activity

To determine if the *VWF* E4BP4 binding element is involved in the repression of transcription in the *VWF*-producing cells, the *VWF* E4BP4 binding motif was mutated in the construct that spans nts -63 to +169, and the effect on promoter activity was assessed in BAECs (Figure 6A). A 5-fold increase in promoter activity was observed with the mutated *VWF* E4BP4 element relative to the wild-type element. Furthermore, the relative promoter activity was 2.6-fold higher than that observed with the full-length -91 to +229 construct, which contains both *trans*-activating GATA elements at nts -80 and +220. This clearly indicates that the *VWF* E4BP4 element is involved in repression of activated *VWF* promoter activity. To confirm the involvement of E4BP4 and the negative response element in repressing basal levels of *VWF* promoter activity, the effect of mutating the E4BP4 binding element was assessed in HepG2 cells. Only a modest relief of repression was observed (Figure 6C). However, since relatively low levels of nuclear E4BP4 are present in HepG2 cells (Figure 5B) and since the extent of repression appears to be related to the amount of nuclear E4BP4 (Figure 4F), the mutant construct was assessed in HepG2 cells that were cotransfected with the E4BP4 recombinant construct (Figure 6D). A significant reduction ($P < .0001$) in *VWF* promoter activity relative to HepG2 cells that were not cotransfected with the recombinant E4BP4 was observed. Furthermore, a significant increase ($P = .0007$) in transcriptional activity was observed when the mutated E4BP4 binding element was compared with the identical construct that contained the wild-type E4BP4 element (Figure 6E).

E4BP4 interacts with the *VWF*-repressive element in *VWF*-expressing and -nonexpressing cells

To conclusively demonstrate that E4BP4 mediates its repressive effect on transcription by interacting *in vivo* with the *VWF* E4BP4 element, a ChIP assay was carried out using chromatin isolated from HUVECs, and differentiated and undifferentiated Dam1 and HepG2 cells. E4BP4 antibodies were used to immunoprecipitate native chromatin from the different cell types, and the presence of

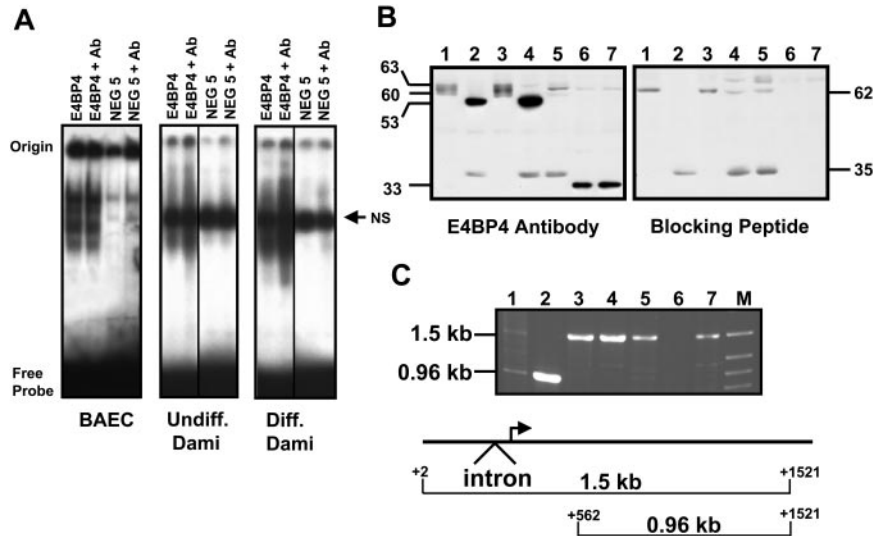


Figure 5. Analysis of E4BP4 in VWF-producing cells. (A) EMSA and supershifts with the E4BP4 antibody (Ab) using 10 µg of nuclear proteins isolated from BAECs, and undifferentiated and differentiated Dami cells. Oligonucleotide probes contain the E4BP4 consensus element (E4BP4) and the *VWF* E4BP4 element (Neg 5). NS indicates the presence of a nonspecific DNA/protein complex, and the free probe and well origin are shown. (B) Western blot analysis of nuclear proteins (20 µg/lane) using the E4BP4 antibody (left panel). The estimated mass in kilodaltons of the different forms of E4BP4 is indicated on the left side of the panel. The right panel shows nonspecific bands present when the E4BP4 antibody is preincubated with the blocking peptide. Lane 1 shows HepG2 transfected with E4BP4; lane 2, 10 µg BAECs transfected with E4BP4; lane 3, HepG2; lane 4, BAECs; lane 5, HUVECs; lane 6, undifferentiated Dami; and lane 7, differentiated Dami. (C) Reverse-transcribed and PCR-amplified RNA isolated from the following: lane 1, BAECs; lane 2, HUVECs; lane 3, differentiated Dami; lane 4, undifferentiated Dami; lane 5, HUVECs; lane 6, BAECs; and lane 7, HepG2. A DNA size marker (GeneRuler 100 bp DNA Ladder Plus MBI) is in lane 8. Below is a diagrammatic representation of the E4BP4 RNA with the single intron and the translational start site indicated. The amplified fragments and sizes are also shown. Lanes 1 and 2 were amplified using primers E4BP4-nt-562-Sense and E4BP4-nt-1521-Antisense. Lanes 3 to 7 were amplified with E4BP4-nt-2-Sense and E4BP4-nt-1521-Antisense.

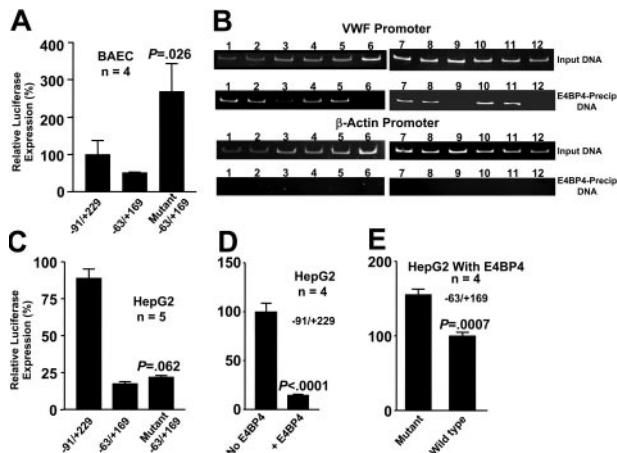


Figure 6. Analysis of a mutated *VWF* E4BP4 binding element and ChIP assays implicate E4BP4 in modulating *VWF* promoter activity. (A) Mean normalized luciferase expression of the mutated and wild-type *VWF* E4BP4 binding element relative to the full-length proximal *VWF* promoter in BAECs. The *P* value from a *t* test to assess for a significant difference between the mutated and the wild-type *VWF* E4BP4 binding motif is indicated on the graph. (B) ChIP analysis of E4BP4-precipitated (precip) chromatin. PCR efficiency is demonstrated by amplifying appropriate fragments from the nonimmunoprecipitated input chromatin. PCR-amplified fragments consist of nt +3 to +225 of the *VWF* promoter. A region of the β -actin promoter²⁵ was used as a negative control. Lanes 1 to 3, 4 to 6, 7 to 9, and 10 to 12 represent chromatin isolated from HUVECs, HepG2, and undifferentiated and differentiated Dami cells, respectively. Lanes 1, 4, 7, and 10 used E4BP4 antibody no. SC9550, and lanes 2, 5, 8, and 11 used E4BP4 antibody no. SC9549X. Lanes 3, 6, 9, and 12 were precipitated with no antibody. (C) Mean normalized luciferase expression in HepG2 of the mutated and wild-type *VWF* E4BP4 binding elements relative to the full-length proximal *VWF* promoter with calculated significant difference between the mutated and the wild-type *VWF* E4BP4 element. (D) Comparison of mean normalized luciferase expression of the full-length proximal promoter in HepG2 cells with and without the E4BP4 expression plasmid. (E) Comparison of mean normalized luciferase expression of the wild-type and mutant E4BP4 binding elements in HepG2 cells cotransfected with the E4BP4 expression plasmid. Error bars represent standard error of the mean.

the *VWF* promoter fragments in the immunoprecipitated fraction was assessed by PCR. The results presented in Figure 6B demonstrate that the *VWF* promoter region spanning +3 to +225 was specifically amplified in the E4BP4-immunoprecipitated chromatin isolated from all 4 cell types. This clearly demonstrates that, in vivo, E4BP4 binds to the negative response element in the *VWF* promoter in cells that actively express VWF and in cells that express only basal levels of VWF.

Discussion

The underlying mechanisms that contribute to cell type-specific expression of the *VWF* gene are complex and not well understood. In our initial analysis, we compared the cell type-specific transcriptional responsiveness of the minimal *VWF* promoter (−91 to +229) in a variety of cell types, relative to the promoterless construct pGL2 Basic. The minimal *VWF* promoter is very ineffective in mediating expression in nonendothelial cells, while in contrast, a 40-fold increase in expression is observed in endothelial cells. Dami cells have been used as a megakaryocyte model and can be induced to differentiate with an associated increase in VWF production.³⁶ In undifferentiated megakaryocytes, the *VWF* proximal promoter activity is about 10-fold greater than in non-VWF-expressing cells, and when these cells are induced to differentiate, promoter activity more than doubles. The complexities of the mechanisms responsible for increased VWF production associated with the induction of Dami cell differentiation are unresolved, and although *VWF* promoter activity increases, the increase in VWF production may also, in part, result from the concomitant polyploidization that occurs with differentiation.²⁷

The role of GATA elements as positive modulators of transcriptional activity in both endothelial cells and megakaryocytes has been described,³⁷⁻⁴³ and the GATA sites at nts −80 and +220, and a

putative GATA element at +53 have been characterized.^{18,21,22} Predictably, in primary endothelial cells, and megakaryocytes, reduction in transcriptional activity is observed in response to removal of the GATA element at nt -80. In HepG2 cells, however, a substantial increase in promoter activity was consistently observed when this GATA element was removed. It is noteworthy that the nucleotide sequences in this region are similar to those in the region of the GATA element at nt +220, and in nonendothelial cells, this sequence interacts with the NFY transcription factor, which represses transcription by recruiting histone deacetylases.^{24,25} It is possible that modifications such as methylation or deacetylation at the GATA element at nt -80 repress transcription, and when these sequences are removed transcription is enhanced in the nonendothelial cells.

The presence of a repressive element at +96/+105 is indicated by the substantial increase in promoter activity that is observed when these sequences are mutated or removed from the *VWF* promoter construct, and this is most pronounced in cells that actively express *VWF*. In HepG2 cells, E4BP4 directly interacts with this element, which appears to repress basal promoter activity (Figure 7A). This inhibition may be accomplished by preventing the formation of a transcription-competent complex.⁴⁴⁻⁴⁷ E4BP4 binds DNA only when it is phosphorylated,^{35,48} so presumably in HepG2 cells the E4BP4 is phosphorylated and this accounts for its larger size on the Western blot. Repression by the *VWF* E4BP4 element is most pronounced in endothelial cells, and yet there is no clear evidence that E4BP4 interacts directly with this element in any of the *VWF*-expressing cells, even though there is an abundance of E4BP4 in the nucleus of these cells. In BAECs, the molecular mass of the protein is smaller than that seen in HepG2 cells, and it is possible that in these cells E4BP4 is not phosphorylated,

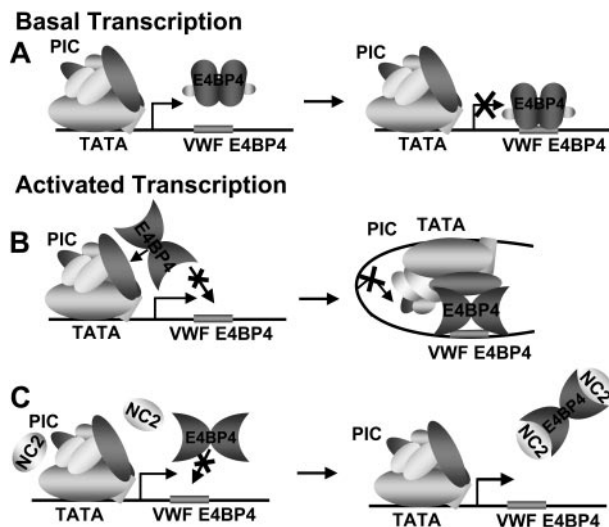


Figure 7. Proposed model of regulation of *VWF* basal and activated transcription by E4BP4. (A) In non-*VWF*-expressing cells, positive regulatory transcription factors are absent, and E4BP4 is phosphorylated, binds directly to the *VWF* E4BP4 element, and inhibits basal levels of transcription.³⁴ (B) In *VWF*-expressing cells, positive regulatory transcription factors are present and productive transcription occurs. E4BP4 is not phosphorylated, or is in some way modified, and cannot directly interact with the *VWF* E4BP4 binding element. Activated *VWF* expression is restrained through coordinated binding of E4BP4 first with an unknown factor, presumably a component of the preinitiation complex (PIC), and then by binding with the *VWF* E4BP4 element. (C) In *VWF*-expressing cells, E4BP4 cannot directly interact with the *VWF* E4BP4 binding element. Instead, the E4BP4 repressor domain is able to sequester negative regulators of transcription, such as NC2, and ultimately facilitate promoter activity.^{31,43} Relative changes in the quantity and/or types of E4BP4 may regulate the activities of these 2 mechanisms in *VWF*-expressing cells.

and may therefore not be able to bind directly to the *VWF*-E4BP4 element to repress active transcription. In Dami cells, the molecular mass of the nuclear E4BP4 is substantially smaller, but analysis of the E4BP4 RNA provides no evidence that any of the different forms of E4BP4 originate from alternative splicing and all have the DNA binding motif. The *VWF* E4BP4 element is clearly involved in repressing *VWF* promoter activity since deletion or mutation of it dramatically increases *VWF* promoter activity. Furthermore, the ChIP assay demonstrates that in both *VWF*-expressing and -nonexpressing cells, E4BP4 does interact with the negative response element *in vivo*. E4BP4 is known to interact with several different nuclear proteins, only one of which, NC2 (Dr1), has been identified.⁴⁴ In addition, the function of the conserved C-terminal region of E4BP4 is unknown. E4BP4 may therefore be part of a coordinated binding complex that must first interact with a DNA-associated nuclear protein before it can interact with the *VWF* E4BP4 binding element (Figure 7B). The presence of different sizes of E4BP4 in the nucleus of the different cell types, and the lack of direct DNA binding of E4BP4 to the *VWF* E4BP4 element only in cells that actively transcribe *VWF*, indicates that E4BP4 uses different mechanisms to repress active and basal transcription. It is interesting to note that in BAECs, HUVECs, and Dami cells there are differences in the amounts and sizes of the various forms of E4BP4, and this may contribute to differences in the level of *VWF* expression between different cell types. Clearly, further studies will be required to elucidate the mechanisms of E4BP4 repression of active transcription.

E4BP4 is known to interact with the TATA binding protein (TBP) NC2. NC2 binds to the TBP to repress transcription, but no complex consisting of all 3 proteins has been identified.⁴⁴ It has been proposed that when E4BP4 does not bind DNA it sequesters NC2, thereby inhibiting its repressive effect on transcription, and that this would effectively enhance transcription (Figure 7C).⁴⁴ In BAECs and Dami cells, there is no direct binding of E4BP4 to the *VWF* E4BP4 binding element, but in BAECs when this element is mutated, not only is there a restoration, but a further enhancement of *VWF* promoter activity. The enhanced promoter activity may result because more E4BP4 is now able to sequester additional negative regulators of transcription, which thereby ultimately enhances promoter activity. In HepG2 cells, where E4BP4 directly interacts with the DNA-binding element, a similar recovery of expression is not seen with the mutated E4BP4 negative response element.

E4BP4 is an inducible transcription factor and appears to play a role in diverse functions, including regulation of circadian rhythm inhibiting apoptosis in pro-B lymphocytes (reviewed in Cowell³³). E4BP4 may play a role in the anti-inflammatory response mediated, in part, by glucocorticoids,⁴⁹ as dexamethasone induces the expression of E4BP4, and furthermore, genes such as Cox-2 and iNOS, which are also inducible by dexamethasone, have E4BP4 binding sites in their promoters. The data we present here further support a role for E4BP4 in regulating proinflammatory gene expression, in this instance mediating a reduction in *VWF* expression following its initial, positive acute-phase response. This possibility suggests that E4BP4 may play an important role in restricting increased *VWF* expression to short-term and transient responses.

In conclusion, this report documents, for the first time, the presence of a *cis*-acting repressor element between nts +96/+105

in the first, noncoding exon of the *VWF* gene. This element interacts with the bZIP transcriptional repressor, E4BP4, and restrains transcription of *VWF* in a cell type-specific manner. This repressor element is the fourth negative regulatory sequence to be

characterized within the *VWF* promoter region. Overall, this information suggests that *VWF* transcription requires tight control, and that this regulatory region has evolved to significantly restrict the likelihood of constitutive protein overexpression.

References

- Sporn LA, Chavin SI, Marder VJ, Wagner DD. Biosynthesis of von Willebrand protein by human megakaryocytes. *J Clin Invest*. 1985;76:1102-1106.
- Jaffe EA, Hoyer LW, Nachman RL. Synthesis of antihemophilic factor antigen by cultured human endothelial cells. *J Clin Invest*. 1973;52:2757-2764.
- Sporn LA, Marder VJ, Wagner DD. Differing polarity of the constitutive and regulated secretory pathways for von Willebrand factor in endothelial cells. *J Cell Biol*. 1989;108:1283-1289.
- Meyer D, Girma JP. von Willebrand factor: structure and function. *Thromb Haemost*. 1993;70:99-104.
- Meyer D, Pietu G, Fressinaud E, Girma JP. von Willebrand factor: structure and function. *Mayo Clin Proc*. 1991;66:516-523.
- Girma JP, Meyer D, Verweij CL, Pannekoek H, Sixma JJ. Structure-function relationship of human von Willebrand factor. *Blood*. 1987;70:605-611.
- Ruggeri ZM. Structure and function of von Willebrand factor: relationship to von Willebrand's disease. *Mayo Clin Proc*. 1991;66:847-861.
- Badimon L, Badimon JJ, Chesebro JH, Fuster V. von Willebrand factor and cardiovascular disease. *Thromb Haemost*. 1993;70:111-118.
- Gill JC, Endres-Brooks J, Bauer PJ, Marks WJ Jr, Montgomery RR. The effect of ABO blood group on the diagnosis of von Willebrand disease. *Blood*. 1987;69:1691-1695.
- Aird WC, Edelberg JM, Weiler-Guettler H, Simmons WW, Smith TW, Rosenberg RD. Vascular bed-specific expression of an endothelial cell gene is programmed by the tissue microenvironment. *J Cell Biol*. 1997;138:1117-1124.
- Aird WC, Jahroudi N, Weiler-Guettler H, Rayburn HB, Rosenberg RD. Human von Willebrand factor gene sequences target expression to a subpopulation of endothelial cells in transgenic mice. *Proc Natl Acad Sci U S A*. 1995;92:4567-4571.
- Wu QY, Drouet L, Carrier JL, et al. Differential distribution of von Willebrand factor in endothelial cells: comparison between normal pigs and pigs with von Willebrand disease. *Arteriosclerosis*. 1987;7:47-54.
- Nagel T, Resnick N, Dewey CF Jr, Gimbrone MA Jr. Vascular endothelial cells respond to spatial gradients in fluid shear stress by enhanced activation of transcription factors. *Arterioscler Thromb Vasc Biol*. 1999;19:1825-1834.
- Keightley AM, Lam YM, Brady JN, Cameron CL, Lillcrap D. Variation at the von Willebrand factor (*vWF*) gene locus is associated with plasma *vWF*:Ag levels: identification of three novel single nucleotide polymorphisms in the *vWF* gene promoter. *Blood*. 1999;93:4277-4283.
- Ferreira V, Assouline Z, Schwachtgen JL, Bahnak BR, Meyer D, Kerbiriou-Nabias D. The role of the 5'-flanking region in the cell-specific transcription of the human von Willebrand factor gene. *Biochem J*. 1993;293(pt 3):641-648.
- Guan J, Guillot PV, Aird WC. Characterization of the mouse von Willebrand factor promoter. *Blood*. 1999;94:3405-3412.
- Janel N, Schwachtgen JL, Bakhshi MR, Barek L, Meyer D, Kerbiriou-Nabias D. Comparison of the 5'-flanking sequences of the human and bovine von Willebrand factor-encoding genes reveals alternation of highly homologous domains with species-specific Alu-type repeats. *Gene*. 1995;167:291-295.
- Jahroudi N, Lynch DC. Endothelial-cell-specific regulation of von Willebrand factor gene expression. *Mol Cell Biol*. 1994;14:999-1008.
- Ardekani AM, Greenberger JS, Jahroudi N. Two repressor elements inhibit expression of the von Willebrand factor gene promoter in vitro. *Thromb Haemost*. 1998;80:488-494.
- Jahroudi N, Ardekani AM, Greenberger JS. An NF1-like protein functions as a repressor of the von Willebrand factor promoter. *J Biol Chem*. 1996;271:21413-21421.
- Schwachtgen JL, Janel N, Barek L, et al. Ets transcription factors bind and transactivate the core promoter of the von Willebrand factor gene. *Oncogene*. 1997;15:3091-3102.
- Cuthbert CD. Expression of Von Willebrand Factor: Transcriptional Regulatory Control and the Influence of Inflammation [PhD thesis/dissertation]. Queen's University, Kingston, ON, Canada; 2000:58-80.
- Schwachtgen JL, Remacle JE, Janel N, et al. Oct-1 is involved in the transcriptional repression of the von Willebrand factor gene promoter. *Blood*. 1998;92:1247-1258.
- Peng Y, Jahroudi N. The NFY transcription factor functions as a repressor and activator of the von Willebrand factor promoter. *Blood*. 2002;99:2408-2417.
- Peng Y, Jahroudi N. The NFY transcription factor inhibits von Willebrand factor promoter activation in non-endothelial cells through recruitment of histone deacetylases. *J Biol Chem*. 2003;278:8385-8394.
- Minami T, Donovan DJ, Tsai JC, Rosenberg RD, Aird WC. Differential regulation of the von Willebrand factor and Flt-1 promoters in the endothelium of hypoxanthine phosphoribosyltransferase-targeted mice. *Blood*. 2002;100:4019-4025.
- Wilhilde CC, Van Dang C, Dipersio J, Kenedy AA, Bray PF. Overexpression of cyclin D1 in the Dami megakaryocytic cell line causes growth arrest. *Blood*. 1995;86:294-304.
- Gimbrone MA Jr. Culture of vascular endothelium. *Prog Hemost Thromb*. 1976;3:1-28.
- Graham FL, van der Eb AJ. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology*. 1973;52:456-467.
- Dignam JD. Preparation of extracts from higher eukaryotes. *Methods Enzymol*. 1990;182:194-203.
- Sierra FA. *A Laboratory Guide to In Vitro Transcription*. Basel, Switzerland: Verlag; 1990.
- Cowell IG, Hurst HC. Transcriptional repression by the human bZIP factor E4BP4: definition of a minimal repression domain. *Nucleic Acids Res*. 1994;22:59-65.
- Cowell IG. E4BP4/NFIL3, a PAR-related bZIP factor with many roles. *Bioessays*. 2002;24:1023-1029.
- Lai CK, Ting LP. Transcriptional repression of human hepatitis B virus genes by a bZIP family member, E4BP4. *J Virol*. 1999;73:3197-3209.
- Chen WJ, Lewis KS, Chandra G, et al. Characterization of human E4BP4, a phosphorylated bZIP factor. *Biochim Biophys Acta*. 1995;1264:388-396.
- Greenberg SM, Rosenthal DS, Greeley TA, Tantravahi R, Handin RI. Characterization of a new megakaryocytic cell line: the Dami cell. *Blood*. 1988;72:1968-1977.
- Deveaux S, Filipe A, Lemarchandel V, Ghysdael J, Romeo PH, Mignotte V. Analysis of the thrombopoietin receptor (MPL) promoter implicates GATA and Ets proteins in the coregulation of megakaryocyte-specific genes. *Blood*. 1996;87:4678-4685.
- Lemarchandel V, Ghysdael J, Mignotte V, Rahuel C, Romeo PH. GATA and Ets cis-acting sequences mediate megakaryocyte-specific expression. *Mol Cell Biol*. 1993;13:668-676.
- Okabe M, Kunieda Y, Shoji M, et al. Megakaryocytic differentiation of a leukemic cell line, MC3, by phorbol ester: induction of glycoprotein IIb/IIIa and effects on expression of IL-6, IL-6 receptor, *mpl* and GATA genes. *Leuk Res*. 1995;19:933-943.
- Ravid K, Doi T, Beeler DL, Kuter DJ, Rosenberg RD. Transcriptional regulation of the rat platelet factor 4 gene: interaction between an enhancer/silencer domain and the GATA site. *Mol Cell Biol*. 1991;11:6116-6127.
- Prandini MH, Martin F, Thevenon D, Uzan G. The tissue-specific transcriptional regulation of the megakaryocytic glycoprotein IIb gene is controlled by interactions between a repressor and positive cis-acting elements. *Blood*. 1996;88:2062-2070.
- Bastian LS, Yagi M, Chan C, Roth GJ. Analysis of the megakaryocyte glycoprotein IX promoter identifies positive and negative regulatory domains and functional GATA and Ets sites. *J Biol Chem*. 1996;271:18554-18560.
- Minami T, Tachibana K, Imanishi T, Doi T. Both Ets-1 and GATA-1 are essential for positive regulation of platelet factor 4 gene expression. *Eur J Biochem*. 1998;258:879-889.
- Cowell IG, Hurst HC. Protein-protein interaction between the transcriptional repressor E4BP4 and the TBP-binding protein Dr1. *Nucleic Acids Res*. 1996;24:3607-3613.
- Mermelstein F, Yeung K, Cao J, et al. Requirement of a corepressor for Dr1-mediated repression of transcription. *Genes Dev*. 1996;10:1033-1048.
- Inostroza JA, Mermelstein FH, Ha I, Lane WS, Reinberg D. Dr1, a TATA-binding protein-associated phosphoprotein and inhibitor of class II gene transcription. *Cell*. 1992;70:477-489.
- Cowell IG, Skinner A, Hurst HC. Transcriptional repression by a novel member of the bZIP family of transcription factors. *Mol Cell Biol*. 1992;12:3070-3077.
- Doi M, Okano T, Yujnovsky I, Sassone-Corsi P, Fukuda Y. Negative control of circadian clock regulator E4BP4 by casein kinase Iepsilon-mediated phosphorylation. *Curr Biol*. 2004;14:975-980.
- Wallace AD, Wheeler TT, Young DA. Inducibility of E4BP4 suggests a novel mechanism of negative gene regulation by glucocorticoids. *Biochem Biophys Res Commun*. 1997;232:403-406.