Role of RNA splicing in mediating lineage-specific expression of the von Willebrand factor gene in the endothelium

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Key Points
- RNA splicing of the first intron of the von Willebrand factor (vWF) gene is essential for expression in the endothelium.
- RNA splicing may play a role in mediating endothelial cell heterogeneity.

Introduction

Von Willebrand factor (vWF) is a plasma protein that mediates platelet hemostatic function and stabilizes coagulation factor VIII. vWF plays a particularly important role in platelet adhesion and aggregation at sites of high shear rates.1 Expression of vWF is restricted to endothelial cells and platelets. vWF is differentially expressed in the vasculature. For example, vWF protein and mRNA levels are higher in veins, compared with arteries,2,3 and in venules compared with arterioles.4 Within the microvasculature, vWF is expressed at particularly low levels in the liver. Expression of vWF also varies between neighboring endothelial cells.5 The elucidation of the mechanisms underlying vWF expression may provide important insights into the molecular basis of vascular diversity.

The structural organization of the mouse, human, and bovine vWF promoter-proximal region is closely related.6-9 The first exon (+1 to +246 in human vWF) encodes the 5′ untranslated sequence. The second exon contains the ATG translational start site. Exons 1 and 2 are separated by an intron (+247 to +1475 in human vWF). To study mechanisms of vWF gene regulation, we have previously used a plug-in-socket approach in which a single copy of a vWF promoter-LacZ cassette is targeted to the Hprt locus of mice. Using this strategy, we showed that a region of the human vWF gene (termed vWF2, between −2182 and the end of the first intron) directed expression in the vasculature of the brain, heart and skeletal muscle, but not in other organs such as aorta, lung, liver, spleen, and kidney.8,9 Nor was expression observed in megakaryocytes. In contrast, an intronless version of vWF2 (between −2182 and +246) coupled to LacZ failed to express in the endothelium of heart and skeletal muscle.10 Expression was rescued by replacement of the first intron of vWF with the second intron of the human β-globin gene.10 These findings suggested one of two possibilities. First, the β-globin intron contains cis-regulatory elements sufficient for rescuing expression. Alternatively, splicing of the first intron of vWF is necessary for expression of LacZ when coupled to the vWF promoter in the Hprt locus.

The goal of this study was to determine the role of RNA splicing in mediating expression of vWF. Using transient transfections, we show that splicing is required for vWF promoter activity in endothelial cells, but not megakaryocytes. Similarly, in mice in which LacZ has been knocked into the endogenous vWF locus, the loss of the first intron abrogated reporter gene expression specifically in endothelial cells, whereas the introduction of heterologous introns reversed this effect. Together, these findings implicate a role for intronic splicing in mediating lineage-specific expression of vWF in the endothelium.

Methods

Plasmid constructions

Generation of vWF and Tie2 promoter-luciferase constructs used in transient transfection assays and of the gene targeting constructs is detailed in supplemental Methods on the Blood website.

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and multiple comparisons by Tukey-Kramer multiple range test. The presence of an upstream first exon containing the 5’UTR (box with dashed outline) and a first intron (dashed line) can only be inferred from their conserved arrangement in higher lower species.

Generation and analysis of Hprt and vWF locus-targeted mice

The generation of Hprt-vWF-βGIVS2-lacZ mice was previously described. The generation of additional mice is detailed in supplemental Methods (supplemental Figure 1). Whole mounts and/or tissue sections from targeted mice were assayed by LacZ staining or vWF immunostaining, as detailed in supplemental Methods. All animal studies were approved by the Animal Care and Use Committee at the Beth Israel Deaconess Medical Center.

Cell culture and transfections

Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza and cultured in endothelial cell medium supplemented with the EGM-2-MV bullet kit (Lonza). Mouse endothelial cells were harvested from the hearts of mice as detailed in supplemental Methods. Transient transfections were carried out as described in supplemental Methods.

Transient transfection assays

Transient transfections were carried out as described in supplemental Methods.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed using a ChIP assay kit (Upstate Biotechnology) according to the manufacturer’s instructions and as detailed in supplemental Methods.

Quantitative reverse transcriptase-polymerase chain reaction

Quantitative real-time transcriptase polymerase chain reaction (qPCR) was carried out as previously described. Primer sequences are shown in supplemental Methods.

Nuclear-cytoplasmic fractionation and protein degradation assays

Subcellular fractionation and protein degradation assays were carried out as described in supplemental Methods.

Statistical analyses

Data are expressed as mean ± standard deviation. The statistical significance of differences of the means was determined by 1-way analysis of variance and multiple comparisons by Tukey-Kramer multiple range test.

Results

Promoter proximal intron-exon structure is conserved across vertebrate species

In human, mouse and bovine vWF genes, the first exon has been shown to encode 5’ untranslated sequences and the second exon begins with the ATG translational start site. Exons 1 (+1 to +246 in human vWF) and 2 are separated by an intron of approximately 1200 bp (+247 to +1475 in human vWF). The exon/intron boundaries are highly conserved between mouse and human, and the splice donor and acceptor sites conform with the GT and AT rule. There is increasing evidence that proximal promoter introns, including 5’UTR introns, play an important role in mediating gene expression. Thus, we asked whether the proximal exon-intron arrangement in humans and mice is conserved across other vertebrate species. Indeed, as shown in Figure 1, the same scheme (5’UTR followed by first intron followed by second exon beginning with ATG initiation codon) is found in chimpanzee, canine, and chicken vWF genes. In the case of the rat vWF gene, the first coding exon begins with ATG. A recent annotation for the zebrafish vWF gene assigned the translational start site to the beginning of exon 1 (of a total of 51 exons). To determine whether the zebrafish gene contains an overlooked 5’ UTR-containing first exon (hence a previously unrecognized first intron), we carried out 5’ rapid amplification of complementary DNA (cDNA) ends using total RNA from zebrafish embryos. We found an additional 98 nucleotides of mRNA preceding the translational start and separated from it in the genome by 2866 nucleotides constituting a plausible intron. Thus, the promoter proximal intron-exon is highly conserved in vertebrates.

Intronic splicing enhances vWF promoter-luciferase reporter gene expression in cultured endothelial cells, but not megakaryocytes

Various lengths of the human vWF promoter with and without introns were coupled to firefly luciferase cDNA in pGL3, and the resulting plasmids were transfected into HUVECs. As an internal control for transfection efficiency, cells were cotransfected with

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Figure 2. Effect of RNA splicing on vWF promoter activity in cultured primary human endothelial cells and MEG-01 cells. (A) HUVECs were transiently transfected with a pGL3 construct containing the human vWF promoter spanning the region between −2182 and +246 (which corresponds to the end of the first exon) without an intron (vWFΔInt) or linked 5’ of the first intron from the human vWF gene (vWF2, the second intron from the human β-globin gene (vWFΔIntGln), the second intron from the mouse DSCR-1 gene (vWFΔIntIn), or the sixth intron from the hagfish factor X gene (vWFΔIntHIn). Alternatively, cells were transfected with a pGL3 construct in which the first intron of the vWF gene was placed upstream of the −2182 and +246 vWF promoter in the correct orientation (Int-vWFΔInt) or in the reverse direction (RInt-vWFΔInt), with a a GL3 construct in which the second intron from the human β-globin gene was placed upstream of the −2182 and +246 vWF promoter in the correct orientation (GInt-vWFΔInt) or with a pGL3 construct containing a 2100-bp fragment of the mouse Tie2 promoter linked in the absence or presence of the first intron of human vWF (Tie2P and Tie2P-vWFΔInt, respectively). (Inset) MEG-01 cells were transiently transfected with a pGL3 construct containing the human vWF promoter spanning the region between −2182 and +246 (which corresponds to the end of the first exon) in the absence or presence of the first intron from the human vWF gene. The results show the mean ± standard deviation of luciferase light units (relative to vWF2) obtained in triplicate from ≥3 independent experiments. *P < .05. n.s., nonsignificant. (B) HUVECs were transfected with vWF2-luc or vWFΔInt-luc constructs. After a 24-hour incubation, total or cytoplasmic (cyto) extract fractions were prepared, RNA was extracted, and luciferase mRNA levels were measured by qPCR. The results show the mean ± standard deviation of luciferase mRNA levels (relative to vWF2) obtained in triplicate from 3 independent experiments. n.s., nonsignificant. (C) HUVECs were transfected with vWF2-luc or vWFΔInt-luc plasmids. After a 24-hour incubation, cells were treated with cyclohexamide (100 μg/mL) and collected at the indicated time points to measure the luciferase activity. Data are presented as percentage relative to 0 hours. n = 3 independent experiments.
splicing stimulates vWF expression in endothelial cells but not megakaryocytes.

RNA splicing has been shown to influence gene expression at multiple steps, including at the levels of transcription, translation, and protein degradation. To investigate the mechanisms underlying reduced luciferase activity in endothelial cells transfected with intronless constructs, we measured luciferase mRNA levels using real-time PCR. These experiments revealed equivalent luciferase mRNA levels in cells transfected with vWF2-luc and vWFΔInt-luc (Figure 2B), suggesting that the enhancing effect of RNA splicing on vWF-luciferase activity is mediated at a post-transcriptional level. Subcellular fractionation revealed comparable levels of cytoplasmic luciferase mRNA in vWF2-luc- and vWFΔInt-luc-transfected endothelial cells (Figure 2B). These data argue against differential nuclear export of spliced and unspliced mRNAs. Finally, in protein degradation assays, we found that luciferase protein stability was comparable in the two transfectants (Figure 2C). Thus, the increased luciferase activity of vWF2-luc (compared with vWFΔInt-luc) is not explained by a reduction in protein degradation.

First intron of the mouse vWF gene is required for β-galactosidase activity in the endothelium of vWF-LacZ knockin mice

We previously demonstrated that expression of a human vWF promoter spanning the region between −2182 and +246 (namely vWF2) directed LacZ expression in blood vessels of the heart and skeletal muscle of Hprt-targeted mice in the presence, but not the absence of the native first intron or the second intron from the human β-globin gene.10 To investigate whether rescue by the β-globin intron was mediated by the presence of enhancer elements, we targeted the Hprt locus of mice with a construct in which the intron was placed upstream of the vWF promoter. At this 5’ position, the β-globin intronic sequence failed to rescue expression in heart and skeletal muscle (supplemental Figure 2). These findings argue against a role for a position-independent enhancer within the β-globin intron.

A limitation of the Hprt locus targeting studies is that a defined length of the human vWF promoter (−2182 to +246, with or without an intron) is inserted into a foreign genomic locus (the Hprt locus) of mice. In contrast to Hprt-targeted vWF2, which drives reporter gene expression in blood vessels of the brain, heart, and skeletal muscle, the endogenous vWF gene is expressed in many other vascular beds. Moreover, in vWF2-LacZ mice, expression of LacZ in the capillaries of heart and skeletal muscle is more widespread than that of vWF. Thus, while the Hprt locus targeting studies provide a valuable tool for dissecting mechanisms of vascular bed–specific gene expression, the extent to which the results may be extrapolated to the endogenous vWF gene is unclear. To circumvent this problem, we targeted LacZ to the endogenous vWF locus. The ATG initiation codon of LacZ was introduced immediately downstream of the first intron (in place of the ATG start site of vWF; Figure 3). Heterozygous mice (vWFΔIntLacZ+/−) were analyzed for LacZ expression. In real-time PCR assays, LacZ mRNA closely paralleled that of the endogenous vWF gene, with the highest levels expressed in the lung, followed by the heart and skeletal muscle (supplemental Figure 3). X-Gal staining of whole-mount organs and tissue sections revealed LacZ expression in multiple vascular beds, including the brain, lung, heart, liver, kidney, and spleen (Figure 4; supplemental Figure 4). The pattern was identical to that observed with the endogenous vWF protein, with the exception that LacZ staining in the glomeruli was less intense compared with the endogenous protein (supplemental Figure 4). These findings suggest that the LacZ knockin mouse is a suitable surrogate for tracking expression of the endogenous vWF gene.

We next generated a LacZ knockin mouse in which the first intron was removed. In this case, the ATG start site of LacZ was placed immediately downstream of the first exon of vWF (Figure 4). The resulting mice were bred to obtain heterozygotes (vWFΔIntLacZ+/−). Whole-mount organs and tissue sections from these animals did not reveal any detectable reporter gene activity in the vasculature, with the exception of the brain where faint LacZ staining was observed in occasional superficial blood vessels (Figure 4; supplemental Figure 5). In contrast, β-galactosidase activity in bone marrow megakaryocytes was unaffected by the loss of the first intron. However, in real-time PCR assays, LacZ mRNA expression in the various organs was identical to that of the intron-containing vWFΔIntLacZ+/+ mouse (Figure 5A-B). To validate these findings, we used magnetic beads to isolate endothelial cells from various organs (heart, lung, brain, and diaphragm) of the vWFΔIntLacZ+/− and vWFΔIntLacZ+/+ mice and assayed for LacZ mRNA levels within 90 minutes of tissue harvest. Consistent with the findings in whole organs, LacZ transcript levels were comparable in endothelial cells from both lines of mice (Figure 5C). In other experiments, endothelial cells from the heart were cultured for 1 to 2 weeks to obtain enough material for ChIP and subcellular fractionation assays. A limitation of primary mouse endothelial cell cultures is that vWF (and in the case of the knockin mice, LacZ) mRNA expression is rapidly downregulated (not shown). Nonetheless, Pol II ChIP revealed equal levels of association with the proximal vWF promoter, providing further support that gene transcription is unaffected by the loss of the first intron (Figure 5D). Moreover, subcellular fractionation assays showed comparable levels of LacZ mRNA in the cytoplasm of endothelial cells from vWFΔIntLacZ+/− and vWFΔIntLacZ+/+ mice (Figure 5E). These findings suggest that the loss of β-galactosidase activity in the absence of the first intron is mediated by a posttranscriptional mechanism that does not involve a defect in nuclear export of mRNA.
LacZ expression in the endothelium, they exhibited quantitative and also rescued expression. Although all 3 introns were able to promote Figure 6). In contrast to the cell culture transfected mice, introns from the in vitro transfection data, introns from the endogenous vWF locus could rescue expression of LacZ. To that knockin mice is rescued by the inclusion of heterologous introns.

We next asked whether the introduction of heterologous introns into the endogenous vWF locus could rescue expression of LacZ. To that end, we chose the same three introns that were tested in transient transfection assays, namely the second intron of the human β-globin gene, the second intron of the mouse DSCR-1 gene, and the sixth β-globin and hagfish genes expressed in endothelial cells (Figure 6; supplemental Figure 6). In contrast to the cell culture findings, the DSCR-1 intron also rescued expression. Although all 3 introns were able to promote LacZ expression in the endothelium, they exhibited quantitative and qualitative differences in their effects. For example, mice carrying the β-globin intron (vWFHInLacZ/+′) demonstrated the most complete rescue, whereas mice carrying the DSCR-1 intron (vWFHInLacZ/) demonstrated the least. Endothelial cells of the atria in vWFHInLacZ/+ mice had much stronger staining compared with vWFHInLacZ/+ mice or mice carrying the hagfish and DSCR-1 introns (supplemental Figure 7). In contrast, mice with the hagfish intron (vWFLacZ/) expressed at much lower levels in arteries compared with the other lines of mice (Figure 7; supplemental Figure 8). Collectively, the data support the conclusion that RNA splicing plays a role in mediating vWF expression in the endothelium.

Discussion

Most genes in higher eukaryotes are interrupted by ≥1 intron. Introns must be precisely removed or spliced from the initial pre-mRNA before nuclear export and translation. The primary role of introns is to generate increased proteomic diversity by alternative splicing. However, introns are also required for optimal gene expression in many systems, including yeast, plants, insects, mammalian tissue culture cells, and transgenic mice. In some cases, intron-dependent gene expression is mediated by transcriptional control elements. For example, previous studies in transgenic mice have implicated a role for intronic enhancers in mediating the expression of vascular endothelial growth factor receptor 2, Tie2, GATA2, and endoglin. In other cases, splicing has been shown to directly stimulate the expression of genes. Our findings are the first to implicate a role for RNA splicing in mediating expression of an endothelial-restricted gene.

The life cycle of eukaryotic mRNA includes a number of steps that include transcription, capping, splicing, polyadenylation, nuclear export, and cytoplasmic translation and degradation. Each of these steps is carried out by tightly coupled multicomponent machines. Intron have been shown to influence gene expression at multiple steps along this pathway. For example, splicing has been reported to enhance mRNA transcription, promote the 3’ end processing/polyadenylation, and reduce mRNA decay. Our finding that luciferase and LacZ mRNA levels were unchanged in the absence of an intron argues against a role for any one of these mechanisms. Other studies have implicated a role for mRNA splicing in mediating increased nuclear mRNA export, however, our experiments in cultured cells failed to reveal an effect of splicing on the nuclear-cytoplasmic distribution of mRNA. Finally, there is increasing evidence that mRNA splicing enhances mRNA translational yield (ie, the number of protein molecules per mRNA molecule). Our finding that intronless vWF promoter-luciferase and vWF promoter-LacZ constructs produced normal levels of mRNA but decreased protein levels in transient transfections and LacZ knockin mice, respectively, indicate that the enhancing effects of splicing in the endothelium are mediated at this level. Increased translational yield may be explained by increased translation of spliced messages or increased stability of encoded polypeptide. We found that the luciferase protein was equally stable whether it was produced from an intronless or intron-containing vWF promoter construct, suggesting that the effects of splicing are secondary to increased translational efficiency.

The splicing reaction results in the deposition of proteins, termed the exon junction complex (EJC), at a conserved position, 20 to 24 nt upstream of exon-exon junctions. These proteins are involved in diverse processes including nuclear export, nonsense-mediated mRNA decay, and translational efficiency. Previous studies suggest that in some cases, EJC deposition contributes to translational enhancement by increasing polysome association of the spliced mRNA. It has been proposed that EJC may promote stable formation of a translationally active mRNP through EJC-dependent recruitment of translation initiation factors, including...
The cDNA sequence of the gene is an important determinant of intron dependence. For example, the second intron from the rabbit β-globin gene was shown to increase expression of a β-globin cDNA fragment 400-fold, the mouse dihydrofolate reductase gene 10- to 20-fold, and the thymidine kinase gene only 2-fold. Another study investigated 10 different human and nonhuman genes for their intron dependence in vertebrate cells. Although all genes tested were expressed more efficiently in the presence of an intron, there were important quantitative differences: enhancement of human β-globin was 35-fold, whereas with 9 other cDNAs, it was 2- to 8-fold. Here, we have shown that the deletion of the first intron of vWF in vWFΔintLacZ/− mice results in a virtual loss of detectable β-galactosidase activity in endothelial cells and that expression is rescued by heterologous introns. Thus, vWF expression in the endothelium appears to be completely dependent on RNA splicing.

Several other factors have been shown to influence the magnitude of intron-dependent effects on gene expression. For example, introns in the S′ region of genes, as exemplified by the first intron of vWF, have a greater enhancing effect than those located in the coding sequence or 3′ UTR. Also, the requirement for intronic splicing depends on the identity of the promoter. For example, introns were shown to be important for expression from immunoglobulin or β-globin promoters, but not from a heat shock promoter. Consistent with these data, we demonstrated that the first intron of vWF was unable to augment expression of the Tie2-luciferase construct in transient transfections. The reason for promoter specificity with respect to intron dependence is unclear. Perhaps, intron-insensitive promoters are more heavily transcribed and thus do not require intronic splicing. Alternatively, it has been proposed that components involved in RNA processing or export may tether to the promoter region.

Finally, in addition to exon sequence context and promoter identity, the effect of mRNA splicing on gene expression is dependent on the sequence of the intron. For example, transgenic mice carrying the human histone H4 promoter couple to chloramphenicol acetyltransferase, a hybrid intron had a much more pronounced effect on expression compared with the SV40 small-t intron. In our study, the native vWF first intron and each of the heterologous introns had different effects on the magnitude of LacZ expression in the vasculature, with the β-globin intron exhibiting the most complete rescue. The mechanism underlying intron-specific effects may include inefficient splicing of some introns due to weak splice sites. Indeed, a previous study demonstrated that the introduction of heterologous introns in dihydrofolate reductase minigenes resulted in less efficient splicing in vitro compared with the native intron. Alternatively, the difference may reflect the presence...
of intron-specific regulatory elements that affect others steps in the mRNA life cycle.

An interesting observation was that the various heterologous introns resulted in qualitatively different patterns of rescue. Most striking was the increased β-galactosidase activity in the endothelium of the atria of the vWFΔIntLacZ/+ mice and the reduced expression of LacZ in the arteries of the vWFΔIntLacZ/+ mice. These data indicate that the identity of the intron determines efficiency of splicing (and/or some other component of the life cycle of vWF mRNA). Our findings have 2 important implications. First, they suggest that the native first intron contributes to the spatial regulation of vWF. Second, they raise the interesting possibility that introns play a role in mediating differential expression of genes in the vasculature.

Our previous studies in transgenic mice and Hprt-targeted mice suggest that the transcriptional mechanisms underlying vWF expression differ between endothelial cells and megakaryocytes. Specifically, each of the human and mouse vWF promoter fragments tested to date has been shown to direct expression in endothelial cells, but not bone marrow.6,9,51 Thus, DNA sequences outside these regions are likely to be required for expression in megakaryocytes. The results of the present study suggest that the role of RNA splicing in mediating vWF expression also differs between endothelial cells and megakaryocytes. Type 1 von Willebrand disease (vWD) is found in persons who have partial quantitative deficiency of vWF. A subgroup of individuals with Type I vWD have reduced plasma vWF but normal platelet vWF:Ag and VWF:Rco (platelet-normal).52-54 It has been assumed, although never proven, that the endothelium of these individuals also has normal vWF:Ag and that the very low plasma levels in these is secondary to defective release of vWF into the plasma and/or increased
clearance of vWF from the plasma. However, our findings raise the interesting possibility that a splicing defect could result in a vWD phenotype in which vWF expression is preferentially lost in endothelial cells.

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Authorship

Contribution: L.Y., L.J., D.B., K.C.S., J.S., D.L., and S.-C.J. designed and performed experiments and analyzed the data; P.O. designed experiments and analyzed the data; and W.C.A. designed and performed experiments, analyzed the data, and wrote the manuscript.

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