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Human von Willebrand factor gene sequences target expression to a subpopulation of endothelial cells in transgenic mice

WILLIAM C. AIRD*†‡, NADIA JAHROUDI§, HARTMUT WEILER-GUETTLER*, HELEN B. RAYBURN*, AND ROBERT D. ROSENBERG*†¶

*Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139; †Department of Medicine, Harvard Medical School, Boston, MA 02115; ‡Hematology–Oncology Division, Department of Medicine, Brigham and Women's Hospital, Boston, MA 02115; and §Department of Radiation Oncology and Pittsburgh Cancer Institute, University of Pittsburgh, Pittsburgh, PA 15261

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ABSTRACT **The present study was undertaken to define the 5′ and 3′ regulatory sequences of human von Willebrand factor gene that confer tissue-specific expression *in vivo*. Transgenic mice were generated bearing a chimeric construct that included 487 bp of 5′ flanking sequence and the first exon fused in-frame to the *Escherichia coli lacZ* gene. *In situ* histochemical analyses in independent lines demonstrated that the von Willebrand factor promoter targeted expression of LacZ to a subpopulation of endothelial cells in the yolk sac and adult brain. LacZ activity was absent in the vascular beds of the spleen, lung, liver, kidney, testes, heart, and aorta, as well as in megakaryocytes. In contrast, in mice containing the *lacZ* gene targeted to the thrombomodulin locus, the 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside reaction product was detected throughout the vascular tree. These data highlight the existence of regional differences in endothelial cell gene regulation and suggest that the 733-bp von Willebrand factor promoter may be useful as a molecular marker to investigate endothelial cell diversity.**

The development of the cardiovascular system is dependent upon a tightly regulated program beginning with differentiation of endothelial cells from mesoderm, followed by their subsequent assembly into vascular channels, and ending with the establishment of organ- and region-specific endothelial cell properties (1, 2). Although the heterogeneity of endothelial cells has been defined at the level of cell structure, function, antigenic composition, and response to environmental stimuli (3–8), the molecular mechanisms that lead to this phenotypic diversity are poorly understood.

The von Willebrand factor (VWF) is a multimeric glycoprotein that mediates adhesion of platelets to damaged endothelium and serves as a carrier for the antihemophilic factor (for reviews, see refs. 9 and 10). The cellular distribution of VWF is restricted to the endothelium, megakaryocytes, and platelets. Histochemical studies of mouse embryos show that the VWF gene is expressed early in vascular development in a limited subset of endothelial cells (2). In adult mammals, VWF is also heterogeneously distributed in different vascular beds and is associated with regional variations in mRNA levels (7, 11–13). The molecular basis of this expression pattern could provide important insights into the genetic programs that govern the production of endothelial cell diversity.

Previous *in vitro* transient transfection studies show that the 2.1-kb upstream domain of the human VWF promoter fails to support high-level specific endothelial cell expression (14, 15), whereas a smaller fragment of the upstream domain of the promoter coupled to the first exon seems to possess this property (16). We have employed a construct containing 487 bp of the 5′ flanking region and the first exon of human VWF fused in-frame to the *Escherichia coli lacZ* gene to produce transgenic mice. The results demonstrate expression of the construct in a subpopulation of yolk sac and adult brain endothelial cells and provide an approach to investigate the molecular basis of endothelial cell heterogeneity.

MATERIALS AND METHODS

Production of Transgenic Mice. To construct plasmid VWFlacZ, a human VWF PCR-generated fragment from positions −90 to +246 was inserted between the *Hin*dIII and *Pst* I sites of pSDKlacZpA (a gift from Janet Rossant, Mount Sinai Research Institute, Toronto) to generate lacZ1K. The *Hin*dIII fragment of HGH-K (17), containing the sequence from positions −487 to −90 of VWF, was then subcloned into the *Hin*dIII site of lacZ1K. The resulting construct was partially digested with *Pst* I, the ends were blunted with the Klenow fragment of DNA polymerase I and religated to obtain a plasmid containing a unique *Pst* I site upstream of the VWF promoter, designated VWFlacZ. The VWF sequence was verified by using Sequenase version 2.0 (United States Biochemical). For injection into fertilized mouse eggs, the VWFlacZ plasmid was digested with *Pst* I and *Kpn* I and the resulting VWF fragment from positions −487 to +246 coupled to the *E. coli lacZ* gene and simian virus 40 poly(A) signal sequence was purified by agarose gel electrophoresis and binding to an NA45 sheet (Schleicher & Schuell). The DNA was resuspended in injection buffer (10 mM Tris·HCl, pH 7.5/0.2 mM EDTA) at 3 μg/ml, passed through a 20 μm (pore size) filter, and injected into the pronuclei of fertilized mouse eggs (17). Offspring were screened for integration of the transgene by Southern blot analysis of *Bam*HI-digested tail genomic DNA with a [^{32}P]dCTP-labeled DNA probe containing the *Pst* I–*Eco*RI fragment of pSDKlacZpA.

Detection of LacZ Activity. Adult mice were anesthetized with i.p. injections of 2.5% (vol/vol) Avertin (17) and then perfused with a fixative solution [2% (wt/vol) paraformaldehyde/1 mM $MgCl_2$/100 mM Pipes, pH 6.9]. The excised tissues were postfixed in fixative solution for 1 hr, washed several times in phosphate-buffered saline (PBS), and then infiltrated overnight with 30% (wt/vol) sucrose/PBS. Samples were subsequently embedded in OCT compound (Miles), quickly frozen on dry ice, and stored at −80°C. Frozen sections (10 μm) were collected in a cryostat, attached to polylysine-coated slides, air-dried, and then incubated with 0.2% glutaraldehyde/1% formaldehyde for 15 min at room temperature. The sections were rinsed in PBS, stained with a 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) staining solution [5 mM potassium ferricyanide/5 mM potassium ferrocyanide/2 mM $MgCl_2$/0.02% Nonidet P-40/0.01% SDS/X-Gal (1 mg/

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Abbreviations: VWF, von Willebrand factor; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; TM, thrombomodulin.

¶To whom reprint requests should be addressed at: Massachusetts Institute of Technology, 68-480, 77 Massachusetts Avenue, Cambridge, MA 02139.

ml) (Boehringer Mannheim) in PBS] for 4–24 hr at 30°C and then counterstained with eosin. Brains that were not embedded in OCT compound were sectioned with a Vibratome, and the resulting 150-μm coronal sections were processed for LacZ activity as outlined above. Bone marrow smears were prepared by aspiration of adult mouse femurs with a 21-gauge needle. After fixing in 0.2% glutaraldehyde/PBS for 25 min, the smears were washed in PBS and examined for *in situ* LacZ activity. The whole mounts of organs, including the tail and aorta, were harvested from nonperfused mice, fixed in 2% paraformaldehyde, and then incubated overnight in X-Gal staining solution. For analysis of embryos, male transgenic mice heterozygous for VWF–*lacZ* were mated to normal FVB female mice. The morning after the appearance of a vaginal plug was designated day 0.5 postcoitus. At various gestational ages, the female mice were sacrificed and embryos were removed from uteri for analysis. The embryos were fixed in 0.2% glutaraldehyde/1% formaldehyde/PBS for 30 min at 4°C, washed in PBS, and then incubated with X-Gal staining solution for 4–24 hr at 30°C. At this point, the yolk sac was either stripped off and attached to polylysine-coated slides for whole mount photography or the embryo was stored in 70% alcohol until paraffin embedding.

Immunohistochemistry. Frozen sections (10 μm) were washed with PBS and saturated with 2% (vol/vol) whole goat serum for 20 min to block nonspecific binding. Sections were then incubated with rabbit polyclonal anti-human VWF antibody (Dako) at a dilution of 1:100 for 1 hr. The sections were washed several times in PBS, incubated with biotinylated goat anti-rabbit IgG secondary antibody for 30 min (Vector Laboratories), rinsed in PBS, and then incubated in 0.3% hydrogen peroxide/0.1% sodium azide for 45 min to inhibit endogenous peroxidase activity. After washing with PBS, the sections were incubated with streptavidin-peroxidase complex (Vector Laboratories), washed again with PBS, and then developed by substrate reaction with diaminobenzidine in the presence of hydrogen peroxide. Methyl green was used as a counterstain. For colocalization of LacZ and VWF, frozen sections were stained overnight with X-Gal staining solution and then processed as above for VWF immunoperoxidase.

RESULTS

Generation of Mice Expressing the VWF–*lacZ* Transgene. We have isolated a 4.07-kb DNA fragment containing the sequence of human VWF from positions −487 to +246 coupled to the *E. coli lacZ* gene and the simian virus 40 poly(A) signal sequence with a *Pst* I/*Kpn* I digestion of the plasmid VWFlacZ (Fig. 1*A*). Microinjection of this fragment into fertilized oocytes resulted in the generation of 12 founder lines of transgenic mice. Eleven of these founder mice gave rise to offspring when mated with wild-type FVB mice. Fig. 1 *B* and *C* shows representative Southern blot analyses of *Bam*HI-digested tail genomic DNA from mice VWF.128 and VWF.131. Copy number was estimated by comparing hybridization signals of probe to transgene and to serial dilutions of a known quantity of linearized VWF–*lacZ* (Fig. 1*C*). Mice VWF.128 and VWF.131 carried four and eight copies, respectively, of the transgene. A third expressing line, termed VWF.111, carried two copies of the transgene (data not shown). The Southern blot analysis of *Eco*RI-digested genomic DNA from lines VWF.128, VWF.131, and VWF.111 revealed a characteristic yet distinct restriction pattern in each line, indicating separate sites of integration within the host genome (data not shown).

LacZ Activity in Adult Tissue. To assay for expression of the transgene in adult mice, frozen sections were prepared from the tissues of offspring of transgenic founders and processed for LacZ activity with X-Gal. *In situ* histochemistry revealed strong LacZ activity (within 3 hr of staining) in blood vessels

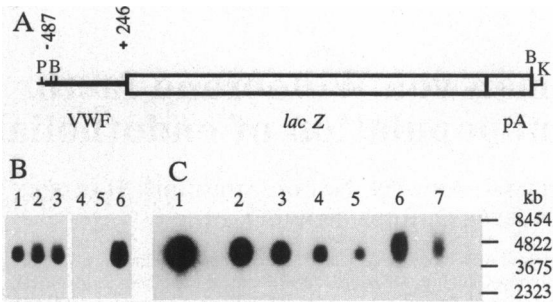

FIG. 1. Construction of the transgene and Southern blot analyses of transgenic mice. (*A*) Structure of the VWF–*lacZ* transgene construct. Plasmid VWFlacZ was digested with *Pst* I and *Kpn* I to obtain the 4.07-kb fragment of the human VWF gene from positions −487 to +246 linked to the *E. coli lacZ* gene and the simian virus 40 poly(A) signal sequence. This fragment was microinjected into fertilized oocytes, resulting in the generation of 12 founder lines of transgenic mice. B, *Bam*HI; P, *Pst* I; K, *Kpn* I. (*B*) Southern blot analyses of transgenic mice from line VWF.128. Samples (10 μg) of *Bam*HI-digested genomic DNA from the tails of VWF.128 mice were electrophoresed on a 1% agarose gel, transferred to a nylon membrane, hybridized with a [^{32}P]dCTP-labeled probe containing the *lacZ*-coding sequence, washed, and exposed to x-ray film. The autoradiogram reveals the expected 4-kb *Bam*HI fragment from heterozygous F_1 offspring (lanes 1–3), homozygous F_2 offspring (lane 6), and nontransgenic littermates (lanes 4 and 5). (*C*) Determination of the copy number. A known quantity of linearized DNA from VWFlacZ equivalent to 32 (lane 1), 16 (lane 2), 8 (lane 3), 4 (lane 4), and 2 (lane 5) copies per diploid genome was electrophoresed on a 1% agarose gel and then compared to the hybridization signal from the Southern blot analysis of 10 μg of *Bam*HI-digested genomic DNA from heterozygous F_1 offspring of lines VWF.131 (lane 6) and VWF.128 (lane 7).

of the brain in two lines, VWF.128 and VWF.131, but not in VWF.111, which showed the X-Gal reaction product in blood vessels of the embryonic yolk sac (see below). LacZ-containing cells were present within the pial vessels, radially penetrating branches, and the microcirculatory beds of the cortical and subcortical areas (Fig. 2 *A–E*). The sections processed for both LacZ activity and immunohistochemical detection of VWF revealed colocalization within endothelial cells (Fig. 2*F*). However, not all brain vessels with immunocytochemically detectable VWF contained the X-Gal reaction product, presumably reflecting variation of transgene expression in endothelial cell subtypes (Fig. 2*D*). In serial sections of the brain from both lines (VWF.128 and VWF.131), LacZ activity was also detected in several regions that did not otherwise possess endogenous VWF: the mammillary bodies, mammillothalamic tract, anterior nucleus of the thalamus, and the periventricular hypothalamus (data not shown). The X-Gal reaction product was not present in brain sections from nontransgenic mice.

Immunohistochemical investigations demonstrated the presence of VWF within endothelial cells of spleen, lung, heart, liver, testis, and kidney (Fig. 3 *A* and *B* shows lung and spleen). In contrast, *in situ* histochemistry of frozen sections from each of the 11 transgenic lines failed to reveal LacZ-containing cells in any of these tissues (Fig. 3 *C* and *D* shows lung and spleen). LacZ activity was also absent in whole mounts of the thoracic and abdominal aorta and in bone marrow aspirates from all transgenic lines (data not shown). Ectopic expression was evident in the follicular sheaths of the tail in the progeny of VWF.128. Finally, the pattern of LacZ staining in mice from line VWF.128 was similar in littermates that were homozygous and heterozygous for the transgene.

To exclude the influence of *lacZ* coding sequences, lacZ mRNA stability or staining artifacts on the distribution of the X-Gal reaction product in endothelial cells, frozen sections were prepared from the organs of adult mice generated with a *lacZ*-targeted thrombomodulin (TM) locus. TM is an integral membrane glycoprotein that is widely expressed throughout the en-

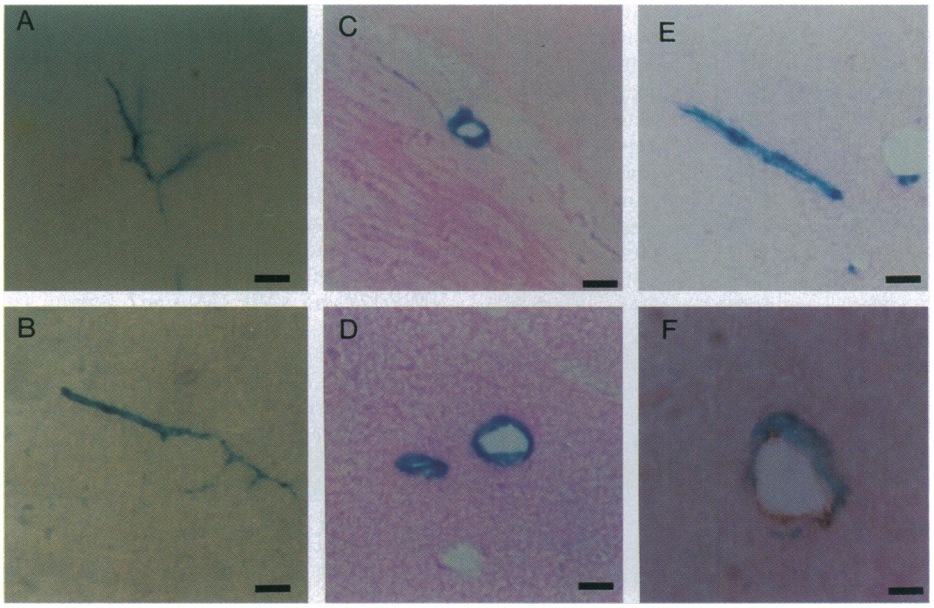

FIG. 2. *In situ* detection of LacZ in adult brain. (*A*) LacZ-staining blood vessels within the cortex in a 150-μm Vibratome coronal section from heterozygous F_2 transgenic offspring of line VWF.128. (*B*) LacZ staining of a blood vessel penetrating from the surface of the brain in a 150-μm Vibratome coronal section from heterozygous F_2 transgenic offspring of line VWF.128. (*C*) X-Gal reaction product of blood vessels in a 10-μm midline brain section from heterozygous F_1 transgenic mouse VWF.128. (*D*) LacZ activity in a similar section from heterozygous F_1 transgenic mouse VWF.131. Note that one of three blood vessels shown in cross-section does not contain detectable LacZ activity. (*E*) LacZ staining of a microvessel in a 10-μm section from the cortex of an F_1 transgenic offspring of line VWF.128. (*F*) Colocalization of the X-Gal reaction product and immunohistochemically detected VWF in a 10-μm midline brain section from an F_1 transgenic offspring of VWF.131. (Bars: *A* and *B*, 62 μm; *C–F*, 23 μm.)

dothelium. Targeted mice were produced by fusing the *E. coli* *lacZ* gene to the promoter of the endogenous TM locus via homologous recombination in embryonic stem cells, injecting the resultant embryonic stem cells into blastocysts to generate chimeric mice, and breeding chimeric animals to pass the targeted gene to the progeny. The production and complete analyses of the above mice will be reported elsewhere. In the *lacZ*-targeted TM animals, LacZ activity was detected in endothelial cells of all organs, including brain, spleen, lung, liver, heart, kidney, and aorta (Fig. 3 *E* and *F* shows lung and spleen).

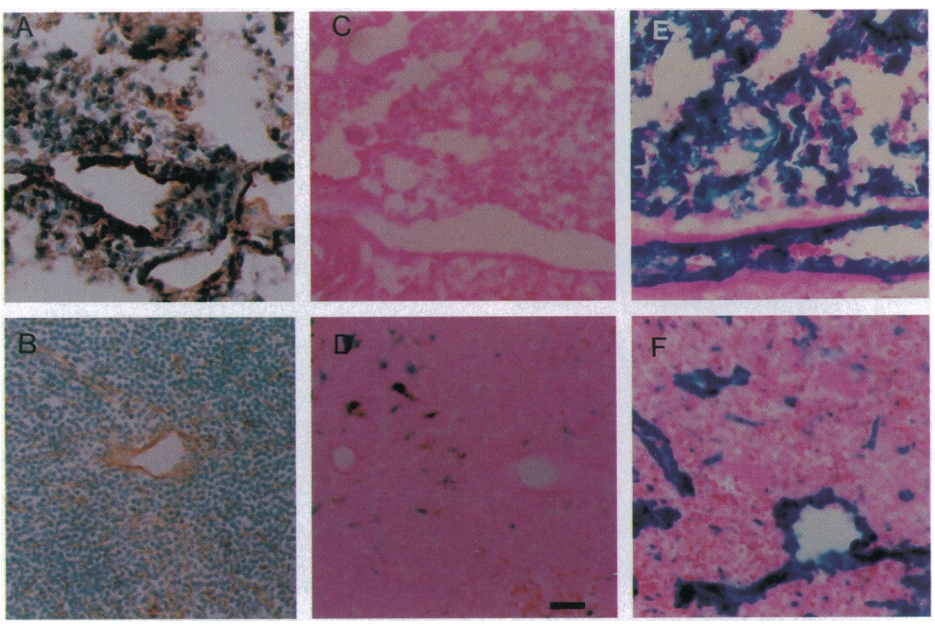

FIG. 3. *In situ* detection of LacZ and VWF in lung and spleen from VWFlacZ and TMlacZ mice. (*A*) Immunohistochemistry of a 10-μm section of the lung shows VWF-positive blood vessels in adult mouse VWF.128. (*B*) Immunohistochemistry of a 10-μm section from the spleen demonstrates VWF-positive blood vessels in adult mouse VWF.128. (*C*) *In situ* histochemistry of a 10-μm section of the lung from adult mouse VWF.128 reveals no detectable LacZ activity in blood vessels. (*D*) *In situ* histochemistry of a 10-μm section of the spleen from adult mouse VWF.128 shows no detectable LacZ activity in blood vessels, two of which are seen in cross-section in the middle of the field. The blue- and green-staining background is present in sections from nontransgenic littermates (data not shown) and likely reflects endogenous LacZ and storage iron, respectively. (*E*) *In situ* histochemistry of a 10-μm section from the lung of adult mouse TMlacZ documents LacZ activity in blood vessels. (*F*) *In situ* histochemistry of a 10-μm section from the spleen of adult mouse TmlacZ demonstrates LacZ activity in blood vessels. (Bar = 63 μm.)

LacZ Activity in Embryos. Transgenic mice from three lines, including VWF.131, did not breed beyond the first generation and were, therefore, not available for analyses of embryos. The embryos from the remaining eight lines were investigated for LacZ activity between days 9.5 and 11.5 postcoitum. In two lines, VWF.128 and VWF.111, whole mount preparations of yolk sacs demonstrated LacZ staining of blood vessels after 4–6 hr of incubation with X-Gal (Fig. 4 *A* and *B*). The paraffin-embedded sections (7 μm) through these embryos showed the highly specific distribution of the X-Gal reaction product in the endothelial cells of the yolk sac blood vessels (Fig. 4 *C* and *D*). The expression of the transgene was not homogenous; some large blood vessels of the yolk sac had no detectable LacZ activity whereas others contained the X-Gal product along part of their length (Fig. 4*A*). In embryos from lines VWF.128 and VWF.111, LacZ-containing cells were also present in structures that did not exhibit endogenous VWF, including the somites and the mesenchyme of the limbs and head.

DISCUSSION

It is widely recognized that endothelial cells play a critical role in the cardiovascular system by regulating hemostatic activity, controlling blood vessel wall tone, and modulating cellular and nutrient traffic. However, it is less often appreciated that endothelial cells of different vascular beds are heterogeneous with regard to structure and function and are thereby able to carry out specialized tasks. For example, the postcapillary high-venuole endothelial cells in lymphoid organs support the binding and migration of lymphocytes via the specific interaction of adhesion molecules with lymphocyte homing receptors (18, 19). The endothelial cells that line the small blood vessels of the brain possess a unique expression pattern of cell surface receptors, transporters, and intracellular enzymes that serve to tightly regulate exchange of solutes between blood and brain parenchyma (20, 21). Distinct endothelial cell phenotypes in other organs, including kidney and lung, have also been documented (22, 23).

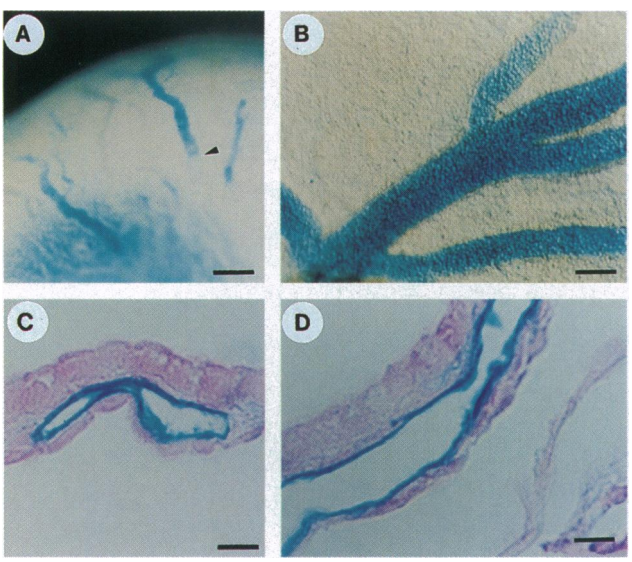

FIG. 4. *In situ* detection of LacZ in the yolk sac. (*A* and *B*) Whole mount preparations of embryonic day 10.5 embryos from line VWF.128 reveal LacZ activity in yolk sac blood vessels. Note the demarcation between LacZ-positive and -negative endothelium within a single large blood vessel of the yolk sac (arrow). (*C* and *D*) Paraffin-embedded 7-μm sections of embryonic day 10.5 embryos from line VWF.128 show that the X-Gal reaction product is located specifically in yolk sac endothelial cells. (Bars: *A* and *B*, 125 μm; *C* and *D*, 47 μm.)

At the present time, little is known about the mechanisms that regulate endothelial cell-specific gene expression *in vivo*. Consequently, we possess few experimental tools with which to examine the molecular basis of endothelial cell heterogeneity. Given that VWF is a marker for differential endothelial cell gene expression, we sought to determine the regulatory regions of the human VWF gene that might direct endothelial cell expression *in vivo*. We generated transgenic mice carrying a chimeric construct that included 487 bp of 5′ flanking sequence and the first exon of the human VWF gene fused in-frame to the coding sequence of *E. coli lacZ*. In two lines, VWF.128 and VWF.131, LacZ activity was detected in endothelial cells of the blood vessels of adult brain, whereas in two independent lines, VWF.128 and VWF.111, the X-Gal reaction product was detected in endothelial cells of the embryonic yolk sac. Surprisingly, LacZ activity was completely absent in all other vascular beds including those that contain high levels of endogenous VWF. This limited pattern of VWF transgene expression in the endothelium contrasts sharply with the homogenous widespread pattern of *lacZ*-targeted TM gene expression. The above data suggest that the differential activity of the transgene is mediated by elements within the human VWF promoter and not by reporter gene sequences or by regional differences in the stability of *lacZ* transcripts. The molecular basis of the differential expression of the VWF transgene presumably involves endothelial subtype-specific variations in regulatory factors, although the role of regional differences in DNA methylation or local chromatin configuration cannot be excluded. Finally, it is noteworthy that line VWF.111 exhibits LacZ activity in embryonic yolk sac endothelial cells but not brain endothelial cells, suggesting that genomic sequences at or near the site of transgene integration influence the pattern of expression and/or that promoter sequences at the 5′ end of the transgene have been deleted. In either case, this observation provides further evidence that gene regulation may differ from one endothelial cell to another.

The absence of LacZ activity in adult tissues outside the brain indicates that DNA sequences distal or proximal to the region between positions −487 to +246 are responsible for directing authentic VWF expression in these organs. The above findings suggest a complex model of tissue-restricted gene regulation in which cis-acting elements required for transcriptional activation of VWF vary among different subpopulations of endothelial cells. This type of mechanism has been proposed in other studies of cell-subtype-specific gene expression (24–26). In one report, the α1 (1) collagen gene was demonstrated to possess different DNA sequences required for expression in fibroblasts of the skin, as compared to the fascia (25). In another investigation, expression of the CD4 gene in transgenic mice was shown to be governed by distinct regulatory elements in separate T-cell subsets (26). The heterogeneous expression pattern of the VWF–*lacZ* transgene suggests that endothelial cell diversity might be generated by regional networks of transacting factors that differentially regulate gene transcription. Indeed, the VWF promoter may serve as a useful molecular marker to dissect these subtype-specific pathways.

It is unclear whether the diversity of adult endothelial cells is generated by endogenous alterations and/or induced by exogenous stimuli from surrounding tissues. *In vitro* investigations using embryonic stem cell cultures suggest that endothelial cell differentiation and early vasculogenesis are genetically predetermined (27). *In vivo* transplant studies using avian species have established that developmental cues for endothelial cell differentiation and overall blood vessel patterning reside in the extracellular environment (1, 28). It is widely believed that a similar set of events regulates differentiation of the mammalian vascular system, although exploration of this issue is limited by the inaccessibility of embryos and the lack of appropriate endothelial cell markers. The strongest support

for the role of exogenous factors in directing regional specialization of the endothelium in mammals is found in investigation of the blood–brain barrier. *In vitro* cell culture and *in vivo* transplant experiments have documented the ability of astroglial cells to induce the blood–brain barrier phenotype in endothelial cells (29–34). However, the nature of the environmental cues and the mechanism by which they mediate blood–brain barrier properties remain poorly defined. The endothelial-subtype-specific expression of VWF–*lacZ* and the ease and sensitivity of the LacZ histochemical assay make the transgene an ideal marker to investigate inducible gene expression. By using transplantation experiments, it should be possible to determine the influence of neuronal signals in inducing VWF transgene expression in otherwise nonexpressing vascular beds. Such studies may provide a framework for the characterization of the extracellular and intracellular signals involved in the differentiation of brain endothelial cells.

During embryogenesis, yolk sac blood vessels arise *de novo* from conversion of mesoderm cells to angioblasts and their subsequent formation of vascular channels. The molecular mechanisms that control these developmental processes remain enigmatic, although recent studies have implicated the pivotal role of receptor tyrosine kinases and their respective ligands (35–38). In the present report, the limited expression of VWF–*lacZ* in the endothelial cells of the yolk sac suggests the potential use of the VWF promoter as a molecular marker for the formation of early blood vessels. The transgenic strain described in this communication should be valuable as a source of marked yolk sac endothelial cells for cell culture studies and for *in vivo* experiments designed to alter the functional development of the vascular system. Moreover, since activation of the promoter appears to be linked to differentiation of yolk sac endothelial cells, identification of the nuclear factors responsible for this switch could provide information about critical events that govern commitment to the endothelial cell lineage.

The production of targeting vectors to investigate the development and physiology of endothelial cells in intact animals constitutes an important goal in vascular biology. The use of transgenic technology to approach this problem has been prevented by our limited understanding of the basis of endothelial-specific gene expression. To the best of our knowledge, no transgene with a tissue-specific promoter has been shown to direct expression of markers to endothelial cells in adult animals. Although the sequence of the VWF promoter from positions −487 to +246 contains only a portion of the information necessary for correct spatial and temporal expression in endothelial cells, the present study suggests that the above objective may be within reach.

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