Vezf1/DB1 Is an Endothelial Cell-specific Transcription Factor That Regulates Expression of the Endothelin-1 Promoter*

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Coordinated gene regulation within the vascular endothelium is required for normal cardiovascular patterning during development and for vascular homeostasis during adulthood, yet little is known about the mechanisms that regulate endothelial transcriptional events. Vascular endothelial zinc finger 1 (Vezf1)/DB1 is a recently identified zinc finger-containing protein that is expressed specifically within endothelial cells during development. In this report, we demonstrate that Vezf1/DB1 is a nuclear-localizing protein that potently and specifically activates transcription mediated by the human endothelin-1 promoter, in a Tax-independent manner, in transient transfection assays. Using a combination of deletion mutagenesis and electrophoretic mobility shift assays, a novel Vezf1/DB1-responsive element was localized to a 6-base pair (bp) motif, ACCCCC, located 47 bp upstream of the endothelin-1 transcription start site. Recombinant Vezf1/DB1 also bound to this sequence, and a 2-bp mutation in this element abolished Vezf1/DB1 responsiveness by the endothelin-1 promoter. Vezf1/DB1 could be identified with a specific antibody in nuclear complexes from endothelial cells that bound to this element. Regulation of endothelin-1 promoter activity by Vezf1/DB1 provides a mechanism for endothelin-1 expression in the vascular endothelium during development and to maintain vascular tone; Vezf1/DB1 itself is a candidate transcription factor for modifying endothelial cell phenotypes in order to appropriately assemble and maintain the cardiovascular system.

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†† The abbreviations used are: ET-1, endothelin-1; Vezf1, vascular endothelial zinc finger 1; AP-1, activator protein 1; HIF-1, hypoxia inducible factor 1; hET-1, human endothelin-1; MEC, myocardial endothelial cells; GST, glutathione S-transferase; GFP, green fluorescent protein; CHIP, carboxyl terminus of Hsc70-interacting protein; CMV, cytomegalovirus; bp, base pair(s); EMSA, electrophoretic mobility shift assay.

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regulated expression of the ET-1 gene. Furthermore, the cooperation between the AP-1 complex and GATA-2 leads to a synergistic increase in trans-activation of ET-1 (11). Last, hypoxia-mediated expression of ET-1 is mediated via trans-activation of the ET-1 promoter by hypoxia-inducible factor-1 (HIF-1) (12). However, the expression of fos/jun and GATA-2 is not restricted to endothelial cells, and the up-regulation of the ET-1 gene via the GATA motif is not limited to GATA-2, as other members of the GATA family, such as GATA-1 and GATA-3, exert a similar effect (11). Because GATA-2, HIF-1, and the Fos and Jun family members are expressed more promiscuously than is ET-1, the action of these factors alone cannot be responsible for the cell type-restricted activation of the ET-1 gene. Therefore, it is possible that the binding of the trans-acting factors to these sites may recruit or otherwise cooperate with additional proteins which are important for cell restricted expression of the ET-1 gene. In any event, a 6-kilobase fragment of the mouse ET-1 promoter confers vascular-specific expression in transgenic mice (13).

Vezf1/DB1 is a recently identified endothelial cell-specific protein. A retroviral trap screen identified a 56-kDa protein expressed specifically in the vascular endothelium, vascular endothelial zinc finger 1 (Vezf1) (14). Vezf1, which is the mouse homologue of a previously identified but incompletely characterized human protein called DB1 (15), is a putative transcription factor that contains 6 Cys2/His2-type zinc finger motifs, as well as a glutamine-stretch and a proline-rich region characteristic of transcriptional activation or repression domains. Vezf1/DB1 is first expressed in the anterior-most mesoderm at day 7.25 post-conception. Expression remains restricted to the vascular endothelium throughout at least day 13.5 and is detectable in endothelial cells undergoing both angiogenesis and vasculogenesis (14). Vezf1/DB1 is therefore an attractive candidate as a potential transcription factor for mediating endothelial cell-specific gene expression, and is expressed in the correct spatial and temporal sequence during embryogenesis to regulate genes critical for endothelial cell differentiation, cardiovascular development, and/or angiogenesis. In an effort to identify transcriptional targets for Vezf1/DB1 in vascular endothelial cells, we have found that Vezf1/DB1 potently trans-activates the human endothelin-1 (hET-1) promoter, and we have characterized a novel Vezf1/DB1-responsive element in the ET-1 5'-flanking sequence.

MATERIALS AND METHODS

Plasmids—Plasmids pGL2-Basic and pGL2-Control contain the firefly luciferase gene (Promega). pGL2-Basic lacks a promoter, whereas pGL2-Promoter expresses luciferase under control of the SV40 promoter (Promega). pGL2-Basic, pGL2-KDR/flk-1, and pGL2-Control contain the firefly luciferase gene of plasmid pGL2-Basic. These constructs share a common 3.5-kb PvuII fragment of the plasmid pGL2-Basic. Plasmids p-204CAT, p-143CAT, p-129CAT, p-95CAT, and p-42CAT, −95/170, pGL2−170, pGL2−143/170, pGL2−129/170, pGL2−95/170, pGL2−42/170, pGL2−42/−170 were created by cloning the BglII fragment of ET-1

Preparation of Nuclear Extracts—All buffers used in the nuclear extract preparation contained the complete EDTA-free protease inhibitor mixture (Roche Molecular Biochemicals) and 5 mM sodium fluoride, 1 mM sodium vanadate, 1 mM sodium molybdate, 0.5 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride. MEC were grown in Dulbecco’s modified Eagle’s medium to confluence in 150-mm plates. Cells were grown at 30 °C for an additional 4 h. Bacteria were lysed by sonication, and GST-Vezf1/DB1 or GST was purified by affinity chromatography on glutathione-Sepharose 4B (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. The protein concentration of recombinant protein was determined by a modified Lowry procedure (DC protein assay, Bio-Rad) and confirmed by 10% sodium dodecyl sulfate-polyacrylamide gel fractionation followed by Coomasie Blue staining.

Preparation of Extracts—Extracts were used for transient transfection experiments. Cells were grown overnight to 70–80% confluence in 6-well plates each containing 2.5 mL of Dulbecco’s modified Eagle’s medium at 37 °C. Cells were transfected with 1.0 μg of pGL2−204/+170, pGL2−204/+170 mut-3, pGL2−143/+170, pGL2−129/+170, pGL2−95/+170, pGL2−42/+170, pGL2−42/−170, and pGL2−42/−170 were created by cloning the BglII fragment of ET-1 promoter fragment extending from −204 bp 5' of the transcriptional start site to position +170 inserted into pGL2-Basic. Plasmids pGL2−204/+170, pGL2−143/+170, pGL2−129/+170, pGL2−95/+170, and pGL2−42/+170 were created by cloning the EcoRI/BglII fragment of plasmids p-204CAT, p-143CAT, p-129CAT, p-95CAT, and p-42CAT, respectively (16), in the appropriate orientation upstream of the luciferase gene of plasmid pGL2-Basic. These constructs share a common 3.5-kb BglII site but differ at the 5’ end located at base pairs −204, −143, −129, −95, −42, and −42, respectively. pGL2-KDR/flk-1 contains a fragment of the KDR/flk-1 promoter from bases −570 to +268, and has been previously described (17). pME18S-DB1 was created by cloning the Pou IV/Not1-digested fragment of IMAGE clone number 2114422 (Research Genetics), which contains the full-length human Vezf1/DB1 cDNA, into mammalian expression plasmid pME18S. p40Tax, a Tax expression plasmid under control of SV40 promoter, was a generous gift from Kuan-Teh Jeang (NIADDK, National Institutes of Health, Bethesda, MD) and has been described elsewhere (18).

Mutagenesis—Site-directed mutagenesis of the hET-1 promoter was performed by polymerase chain reaction with PCR to create the plasmid pGL2−204/+170 mut-3. A DNA fragment containing hET-1 promoter (pGL2−204/+170) was used as a template. The sequence TTACCCCTACTC was mutated to TTATGCTTCAC using the mismatched primers 5’-TACAGGCTTGGTATCCTCTATAGGGGTTC-3’ and 5’-GAACCCCTATAGGTTAAGACGCTGAC-3’. The sequence of the mutated polymerase chain reaction fragment was confirmed by the dideoxy chain termination method.

Cell Culture—The mouse myocardial endothelial cells (MEC), which were a kind gift from Robert Auerbach (University of Wisconsin), have been described elsewhere (19). NIH/3T3 and C2C12 cells were obtained from the Tissue Culture Facility at Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC. These cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum (or calf serum for NIH/3T3 cells), 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Transfection and Luciferase Assays—MEC, C2C12, and NIH/3T3 cells were used for transient transfection experiments. Cells were grown overnight to 70–80% confluence in 6-well plates each containing 2.5 mL of Dulbecco’s modified Eagle’s medium at 37 °C. Cells were transfected with 1.0 μg of pGL2−204/+170, pGL2−204/+170 mut-3, pGL2−143/+170, pGL2−129/+170, pGL2−95/+170, pGL2−42/+170, pGL2−42/−170, and pGL2−42/−170 were created by cloning the BglII fragment of ET-1 promoter fragment extending from −204 bp 5' of the transcriptional start site to position +170 inserted into pGL2-Basic. Plasmids pGL2−204/+170, pGL2−143/+170, pGL2−129/+170, pGL2−95/+170, and pGL2−42/+170 were created by cloning the EcoRI/BglII fragment of plasmids p-204CAT, p-143CAT, p-129CAT, p-95CAT, and p-42CAT, respectively (16), in the appropriate orientation upstream of the luciferase gene of plasmid pGL2-Basic. These constructs share a common 3.5-kb BglII site but differ at the 5’ end located at base pairs −204, −143, −129, −95, −42, and −42, respectively. pGL2-KDR/flk-1 contains a fragment of the KDR/flk-1 promoter from base pairs −570 to +268, and has been previously described (17). pME18S-DB1 was created by cloning the Pou IV/Not1-digested fragment of IMAGE clone number 2114422 (Research Genetics), which contains the full-length human Vezf1/DB1 cDNA, into mammalian expression plasmid pME18S. p40Tax, a Tax expression plasmid under control of SV40 promoter, was a generous gift from Kuan-Teh Jeang (NIADDK, National Institutes of Health, Bethesda, MD) and has been described elsewhere (18).
incubated on ice for 10 min. Cells were vortexed for 10 s, and centrifuged at 5000 rpm for 30 s. The supernatant was discarded and the pellet was resuspended in 80 μl of extraction buffer (20 mM HEPES, pH 7.9, at 4 °C, 25% glycerol, 0.5 mM KCl, 0.75 mM MgCl₂, 0.2 mM EDTA). The mixture was incubated for 45 min at 4 °C for nuclear extraction followed by ultracentrifugation and collection of the supernatant. The supernatant was dialyzed for 30 min in dialysis buffer (20 mM HEPES, pH 7.9, 20% glycerol, 0.1 mM KCl, 0.2 mM EDTA), aliquoted, and snap frozen at −80 °C.

Antibodies—Polyclonal antibodies were generated in rabbits against a peptide (MENWWTAFPFLQAEHC) corresponding to a conserved region of Vezf1/DB1 coupled to keyhole limpet hemocyanin by standard procedures as previously described (20). The IgG fraction was purified from whole immune sera using Affi-Gel 10 (Bio-Rad) packed in a purification column as described in Affinity Purification of Antibodies from Crude Serum (www.protocol-online.net/immuno/antibody/antibody_purification.htm).

Electrophoretic Mobility Shift Assay (EMSA)—EMSA was performed as previously described (21). The probe consisted of annealed synthetic 54-mer complementary oligonucleotides corresponding to −95 to −42 of the hET-1 5′-flanking sequence (13) or a portion of the aforementioned hET-1 5′ flanking sequence corresponding to −95 to −67 (5′ probe), −76 to −57 (middle probe), or −66 to −42 (3′ probe) as shown in Fig. 5A. The synthetic oligonucleotides used for competition were the same oligonucleotides or mutated versions of the 5′ probe as shown in Fig. 6A. A typical binding reaction contained 75,000 cpm of DNA probe, 1.0 μg of poly(dI-dC)-poly(dI-dC), 5 μl of dialysis buffer (20 mM HEPES, pH 7.9, 10% glycerol, 100 mM KCl, and 0.2 mM EDTA), 0.6 μl of bovine serum albumin (10 mg/ml), 10 μg of nuclear extract, or 400 ng of recombinant Vezf1/DB1 in a final volume of 25 μl. The reaction mixture was incubated at room temperature for 25 min and fractionated on a 4% polyacrylamide gel in 1 × Tris glycine buffer. To determine the specificity of the DNA-protein complexes, we performed competition assays using a 50-fold molar excess of the unlabeled wild-type oligonucleotides (specific competitors), mutated oligonucleotides, or an unrelated CT/GC-rich double-stranded oligonucleotide of comparable length (5′-CCCCACCT-GTTGGCAGTTC-3′) as a nonspecific competitor. To characterize specific DNA-binding proteins, we incubated nuclear extracts with affinity purified polyclonal anti-Vezf1/DB1 antibody or a similarly prepared antibody to the unrelated sequence QEKILISEENL, or anti-GST antibody (Sigma) for 3 h at 4 °C before adding probe.

RESULTS

Vezf1/DB1 Is an Endothelial Cell-specific Zinc Finger Protein That Localizes to the Nucleus—Analysis of primary amino acid sequence of Vezf1/DB1 demonstrates that it contains 6 Cys2/His2-type zinc finger motifs and a proline-rich region characteristic of transcriptional activation or repression domains (14, 15), suggesting that Vezf1/DB1 is likely to function as a transcription factor to regulate endothelial cell-specific gene expression. As a first step toward determining the function of this protein, we characterized the intracellular localization of Vezf1/DB1. We expressed a fusion of full-length Vezf1/DB1 with GFP by transient transfection of MEC to localize expression of Vezf1/DB1 within the cell. As controls, we also expressed GFP alone and GFP fused to CHIP, a protein that we have previously shown to localize exclusively to the cytoplasm in this assay (20). Consistent with our previous results, GFP-CHIP localized to the cytoplasm (Fig. 1, middle panels), and GFP alone exhibited diffuse expression throughout the cell (lower panels). However, GFP-Vezf1/DB1 localized exclusively to the nucleus (upper panels). These results are consistent with previous assumptions that Vezf1/DB1 is a nuclear-localizing transcription factor, and led us to search for potential endothelial cell-specific transcriptional targets for this protein.

Vezf1/DB1 Specifically Trans-activates the hET-1 Promoter in Transient Transfection Assays—In order to identify potential transcriptional targets of Vezf1/DB1, we tested known endothelial cell-specific promoters to determine if they are trans-activated by Vezf1/DB1 in transient transfection assays. In our initial experiments, the reporter plasmids pGL2-Basic, pGL2-Promoter, pGL2-KDR/flk-1, or pGL2-204/+170 (expressing a fragment of the hET-1 promoter) were co-transfected with Vezf1/DB1 into MEC. Co-transfection of Vezf1/DB1 had no effect on activity of the SV40 promoter (present in pGL2-Promoter) or on the endothelial cell-restricted promoter KDR/flk-1 (17) (Fig. 2A). In contrast, Vezf1/DB1 significantly trans-activated the hET-1 promoter, another endothelial cell-restricted promoter (16), by ~40–50-fold. The trans-activation of hET-1 promoter was dose-dependent and occurred over a broad range of plasmid concentrations (Fig. 2B). These results demonstrate that Vezf1/DB1 functions as a transcriptional activator and provide a rationale for further characterization of the mechanisms of trans-activation of the hET-1 promoter by Vezf1/DB1.

Tax Does Not Potentiate Vezf1/DB1 Responsiveness by the ET-1 Promoter—The human homologue DB1 was originally cloned via potential interactions with a CT/GC-rich region in the interleukin-3 promoter (15). This same CT/GC-rich region in the interleukin-3 promoter has been defined as a responsive element for Tax-mediated trans-activation (22). Although DB1 did not have any direct transcriptional effects on the interleukin-3 promoter, DB1 augmented Tax-dependent transcriptional activity of interleukin-3 promoter by ~3-fold in a phorbol ester-dependent manner (15). In order to determine whether the mechanisms of trans-activation of the hET-1 promoter by Vezf1/DB1 were different or similar to its effects on the interleukin-3 promoter, we asked whether similar functional interactions between Vezf1/DB1 and Tax occurred to modulate transcription of the hET-1 promoter. We co-transfected MEC with p40^Tax and Vezf1/DB1. As expected, Vezf1/DB1 trans-activates the hET-1 promoter; however, in contrast to its effects on the interleukin-3 promoter, co-transfection of Tax caused a dose-dependent repression of the transcriptional activity of Vezf1/DB1 (Fig. 3). These experiments emphasize that the effects of Vezf1/DB1 on hET-1 promoter activity are distinct in several respects from previous experiments using the interleukin-3 promoter, in which Vezf1/DB1 seemed to function primarily as a transcriptional co-activator for Tax rather than as a direct trans-activator.
the specific sequences that are Vezf1/DB1-responsive. Deletion constructs from base pairs –204 to –95 were equally trans-activated by Vezf1/DB1, whereas further deletion to bp –42 totally abolished Vezf1/DB1-responsiveness (Fig. 4). These experiments thus localize the Vezf1/DB1-responsive element to a 54-bp sequence between –95 and –42 within the hET-1 5′-flanking sequence.

Characterization of a Nuclear Protein-binding Activity within the Vezf1/DB1 Response Element—Having identified the putative Vezf1/DB1-responsive element within the hET-1 promoter via transient transfection assays, we sought to refine the location of this response element, and to determine whether nuclear proteins in general, and Vezf1/DB1 specifically, bound to these sequences. As a first step, we determined whether MEC contain nuclear binding activities that will associate with the fragment from –95 to –42 of the hET-1 5′-flanking sequence by EMSA. MEC were a convenient cell type to utilize for these experiments, as we found them to express Vezf1/DB1 constitutively (data not shown). A 32P-labeled synthetic probe containing base pairs –95 to –42 or portions of it consisting of a series of overlapping oligonucleotides from base pairs –95 to –67 (5′ probe), –76 to –57 (middle probe), or –66 to –42 (3′ probe) were used to probe MEC nuclear extract (Fig. 5A). Incubation of radiolabeled double-stranded oligonucleotide consisting of base pairs –95 to –42 with nuclear extract from MEC identified several DNA-protein binding complexes (Fig. 5B), 3 of which could be specifically competed away with a 50-fold molar excess of the unlabeled full-length (FL) or 3′ probe competitors but not by an excess of the 5′ or middle nonspecific competitors. This experiment indicated that specific DNA/protein interactions could be detected within the Vezf1/DB1 response element, and that these interactions most likely occurred within the 3′ region of this sequence.

To further characterize nuclear protein binding within the Vezf1/DB1 response element, the 5′, middle, or 3′ probes were separately radiolabeled and used in a similar EMSA experiment (Fig. 5C). Only nonspecific binding activities were observed with the 5′ and the middle probes. However, the 3′ probe showed both specific and nonspecific binding activities that essentially recapitulate the binding activities seen in Fig. 5B. (We have labeled these binding activities A, B, and V. The fastest migrating binding activity, V, is variably present in our reactions and likely represents a labile activity or a degradation product derived from a component of one of the slowly migrating activities.) In addition to confirming the results of Fig. 5B, this experiment further localizes nuclear protein binding to the distal portion of the 3′ probe, a 16-bp region between bps –57 to –42 of the hET-1 5′-flanking sequence.

ACCCCC Is the Minimal Binding Site within the Vezf1/DB1 Response Element—In an effort to define the exact nucleotides necessary for Vezf1/DB1 binding within the hET-1 5′-flanking sequence, we made a series of 2- and 4-bp mutations in the minimal putative binding motif within the 3′ probe (Mut-1 to Mut-7, Fig. 6A) and tested their ability to compete for binding with the wild-type radiolabeled 3′ probe. Mutants 2, 3, and 4 failed to compete for this binding activity (Fig. 6B), whereas mutants 1, 5, 6, and 7 competed with efficiency nearly equal to that of the wild-type probe, indicating that ACCCCC is the minimal sequence required to recapitulate the binding activity observed in endothelial cell nuclear extracts.

Vezf1/DB1 Interacts Directly with the Minimal Binding Element in the Vezf1/DB1 Response Element—If Vezf1/DB1 trans-activates the hET-1 promoter by direct interactions with sequences within the hET-1 5′-flanking sequence, we can then hypothesize that one or more of these specific binding activities contains endogenous Vezf1/DB1. To explore this hypothesis, we
tested the ability of recombinant Vezf1/DB1 (expressed in bacteria as a GST fusion) to bind to the minimal binding element by EMSA (Fig. 6C). This experiment shows that recombinant Vezf1/DB1 can, indeed, form a DNA-protein complex with the 3′ probe, which can be competed away with an excess of the wild-type unlabeled probe. In addition, the binding determinants for binding with recombinant Vezf1/DB1 were identical to those of nuclear extract, based on competition experiments with the mutated oligonucleotides. These experiments indicate that Vezf1/DB1 can indeed bind directly to a specific sequence within the hET-1 5′-flanking sequence; in addition, they argue strongly that the binding activity of nuclear extract consists, in part, of endogenous Vezf1/DB1, and provide a rationale for examining whether ACCCCC is the bona fide Vezf1/DB1 response element.

**ACCCCC Is the Vezf1/DB1 Response Element in the hET-1 5′-Flanking Sequence**—To test the hypothesis that the sequence ACCCCC is indeed the Vezf1/DB1 response element, we mutated 2 bp in this sequence (analogous to Mut-3 in the EMSA experiments, Fig. 6A) in the context of the wild-type ET-1 promoter and tested the ability of Vezf1/DB1 to trans-activate this mutated promoter in transient transfection assays. In contrast to the wild-type ET-1 promoter, the mutated promoter was resistant to trans-activation by Vezf1/DB1 (Fig. 7, A and B). Thus, we can conclude that the minimal sequence ACCCCC is indeed the Vezf1/DB1 response element in the human ET-1 promoter.

**Vezf1/DB1 Is Present in DNA-Protein Complexes Formed in Nuclear Extracts from Endothelial Cells**—We typically see more than one specific complexes by EMSA with the ACCCCC element (Figs. 5 and 6, arrows). The similarity between the binding determinants for nuclear extracts and recombinant Vezf1/DB1 indicates that one or more of these activities is likely to contain Vezf1/DB1 itself. The diversity of the binding activities in endothelial nuclear extracts may reflect one or more of the following: (i) multiprotein complexes containing Vezf1/DB1; (ii) additional nuclear proteins binding the same element; or (iii) proteolytic products producing different gel...
shift patterns. To discern among these possibilities, we have raised affinity purified rabbit polyclonal antibodies against a 15-amino acid Vezf1/DB1 peptide (MEANWTALFQVAHEC). As a more precise test of the component of DNA-protein complex, we preincubated nuclear extracts from MEC with anti-Vezf1/DB1 antibody prior to the EMSA binding reactions. For-
In this report, we identify Vezf1/DB1 as an endothelial cell-specific transcription factor. We also characterize a logical transcriptional target, and the DNA response element through which this target is chosen. Vezf1/DB1 potently trans-activates the hET-1 5′-flanking sequence through a novel response element, ACCCCC, to mediate high level transcriptional activity of this promoter. Proteins from endothelial cell nuclear extracts bind specifically to this essential response element, and endogenous Vezf1/DB1 exists as a component of this binding activity. These results provide convincing evidence that Vezf1/DB1 acts as a transcription factor and that ET-1 is a transcriptional target for this protein. In addition, these studies provide a rational explanation for inducible expression of ET-1 specifically within the vascular endothelium.

Vezf1/DB1 was first identified by screening an expression library with a GC-rich Tax-responsive element within the interleukin-3 promoter (15). Recombinant Vezf1/DB1 could bind to this GC-rich sequence by EMSA; however, Vezf1/DB1 by itself was not able to trans-activate the interleukin-3 promoter, although it did modulate Tax-mediated trans-activation in a phorbol ester-dependent fashion. We have similarly seen low-affinity interactions between Vezf1/DB1 and GC-rich regions within the KDR/flk-1 promoter in EMSA experiments; yet Vezf1/DB1 does not trans-activate the KDR/flk-1 promoter (Fig. 2A). Therefore, we suspect these low affinity interactions with GC-rich sequences may not be physiologically significant. In contrast, the highly specific interactions identified between Vezf1/DB1 and the ACCCCC motif result in potent trans-activation of the ET-1 promoter. These observations, in conjunction with the overlapping expression pattern of Vezf1/DB1 and ET-1 during development (4, 14), makes regulation of ET-1 by Vezf1/DB1 a more plausible physiologic interaction to regulate gene expression in vascular endothelial cells. The ACCCCC sequence characterized in these studies as the Vezf1/DB1-binding site and response element does not exactly correspond to any previously identified transcription factor-binding sites, as determined by searches of the TRANSFAC database. However, it is interesting to note that MAZ, which is closely related to Vezf1/DB1 and also contains 6 highly similar zinc fingers of the Cys2/His2 type, binds to response elements containing stretches of G or C residues (23, 24), suggesting that this family of transcription factors may have particularly high affinity for homopolymeric stretches of G/C residues. Identification of a defined Vezf1/DB1-binding site will aid in the discovery of other Vezf1/DB1-responsive genes within the vascular endothelium.

The studies presented here do not specifically address how Vezf1/DB1 activity itself is regulated, and what the consequences of differences in Vezf1/DB1 activity might mean with respect to ET-1 expression, although some interesting hypotheses can be generated based on our observations and those of others. ET-1 expression in endothelial cells is known to be dependent on Rho GTPase activity, and Rho signaling itself can directly activate the ET-1 promoter (25). This effect may have particular importance with respect to modulating vasoconstrictive, migratory, and proliferative effects of cells that are ET-1-responsive, such as smooth muscle and neural crest cells, especially since Rho signaling pathways down-regulate endothelial nitric-oxide synthase (26), which opposes the actions of ET-1 within the vasculature. The relationship between ET-1 expression and Rho signaling is significant because physical interactions have been demonstrated between Vezf1/DB1 and the fraction of prenylated RhoB that is localized to the nucleus (27). It is tempting to speculate that Vezf1/DB1 serves, at least in part, to mediate Rho-dependent signaling events, such as ET-1 expression, at the transcriptional level in endothelial cells.

Although previous data provide few insights into the function of Vezf1/DB1, the expression of this gene during development argues strongly for a specific role in vascular development. The expression of Vezf1/DB1 overlaps significantly during development with KDR/flk-1 (14), a receptor for vascular endothelial growth factor and a marker for endothelial cells and their precursors during development in the mouse (28). This would suggest: 1) that Vezf1/DB1 may be upstream of
KDR/flk-1 by up-regulating its expression; 2) that Vezf1/DB1 is induced in KDR/flk-1-expressing cells; or 3) that KDR/flk-1 and Vezf1/DB1 are regulated in parallel, possibly through similar mechanisms. We have found no evidence for the first possibility, that Vezf1/DB1 regulates expression of KDR/flk-1 (Fig. 2A). Instead, we find that Vezf1/DB1 trans-activates ET-1, which appears later than KDR/flk-1 during vascular development. Deficiency of ET-1 during development results in branchial arch abnormalities, leading to defects in branchial arch artery development and subsequent malformation of the great vessels and cardiac outflow tract abnormalities, as well as characteristic craniofacial abnormalities (4, 5). If the effects of Vezf1/DB1 on ET-1 expression observed in the present studies are representative of regulatory events that are critical in endothelial cells during vascular development, then absence of Vezf1/DB1 in mice should phenocopy, at least partially, ET-1 expression observed in the present studies (Fig. 9). Although cell type-specific gene regulation is beginning to be understood for many lineages, such as skeletal myocytes (29), there is still little known about the transcriptional mechanisms that regulate cell type-specific gene expression in the vascular endothelium. For example, KDR/flk-1 is a well characterized marker for vascular endothelial cells during development, and studies have demonstrated the importance of a variety of transcription factors in control of its expression, including Sp1 (31), TFII-I (21), and GATA proteins (32, 33). However, none of these proteins by themselves account for the restricted pattern of KDR/flk-1 expression during development. Similarly, GATA-2, AP-1, and HIF-1 have all been implicated in the transcriptional regulation of ET-1 (9, 10, 12), yet the means by which ET-1 expression is induced specifically within the vascular endothelium is still not explained.

Several reports have implicated Ets family members in endothelial development and cell type-specific gene regulation (34, 35); however, most of these proteins are expressed in many lineages other than endothelial cells, so Ets proteins are likely required to cooperate with factors that are expressed in a more restricted fashion to mediate endothelial cell gene regulation. The helix-loop-helix transcription factor SCL/tal-1 has been implicated in endothelin-specific transcriptional events as well. Although SCL/tal-1 is dispensable for endothelial cell specification but required for generation of all hematopoietic lineages (36–38), a role in endothelial pattern formation has been attributed to SCL/tal-1 (39). These data would suggest that SCL/tal-1 functions to direct endothelial cell gene expression in early stages of development, although this conclusion has been drawn into question by the demonstration that these effects of SCL/tal-1 are DNA-binding independent (40). In any event, endothelial transcriptional targets for SCL/tal-1 have not been well characterized, and the prominent role of SCL/tal-1 in hematopoietic transcriptional events indicates that SCL/tal-1 cannot, by itself, explain endothelial cell type-specific gene expression. In contrast, the identification of Vezf1/DB1 as a developmentally regulated, endothelial specific protein (14), and our demonstration here that it is a nuclear localizing protein that functions as a transcriptional activator, indicates that Vezf1/DB1 may serve as an important missing link in our understanding of endothelial cell type-specific gene regulation. Although further studies will be necessary to determine the range of endothelial cell genes that are regulated by Vezf1/DB1, and the functional role of this protein in the modulation of endothelial cell phenotypes, our studies support the hypothesis that Vezf1/DB1, in cooperation with other transcription factors yet to be determined, assists in the cellular process of determining the complement of genes that are expressed within the vascular endothelium.

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**REFERENCES**


13. Harats, D., Kurihara, H., Belloni, P., Oakley, H., Ziober, A., Ackley, D., Cain,