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.

The endothelium maintains vascular integrity during adulthood by regulating vascular tone, lymphocyte trafficking, vessel growth, and hemostasis (1). In particular, the vascular endothelium modulates vascular tone and blood pressure through the coordinated production of potent vasoactive molecules. In addition, the endothelium directs formation of the vascular system during development through its role in the processes of vasculogenesis (development of blood vessels from angioblasts in situ) and angiogenesis (sprouting of new vessels from existing vessels) (2). Whereas endothelial mechanisms during development and in adulthood may seem discrete, there is now strong evidence that they overlap considerably, and that molecules required for normal vascular development may contribute to vascular homeostasis in adulthood, and vice versa.

Endothelin-1 (ET-1), a 21-amino acid peptide originally isolated from porcine endothelial cells (3), provides one example of an endothelial cell protein that is critical both for cardiovascular development in the embryo and for vascular homeostasis in the adult. ET-1, which is expressed primarily in endothelial cells and also epithelial cells of the branchial arches during development (4), is required for the development of neural crest-derived tissues arising from the branchial arches, such as the great vessels, the ventricular septum, and craniofacial structures (5). These effects seem to be mediated via interactions with the endothelin-A receptor, which is expressed in neural crest cells. Loss of ET-1 signaling leads to apoptosis of post-migratory neural crest-derived mesenchymal cells, with resultant cardiovascular defects such as ventricular septal defects, truncus arteriosus, and interruption of the aortic arch.

In addition to its function as a critical developmental signal for the cardiovascular system, ET-1 has potent and complex effects on the adult vasculature. ET-1 has direct vasoconstrictor effects on vascular smooth muscle cells, and sensitizes smooth muscle cells to the effects of angiotensin II and norepinephrine (6). ET-1 also stimulates the release of aldosterone and nitric oxide, the latter effect indicating that the vasoconstrictor effects of ET-1 may be most pronounced when endothelial function is compromised. The effects of ET-1 are linked pathophysiologically to the development of hypertension, cardiac hypertrophy (7), and ischemic heart disease (8).

Given the bifunctional role of ET-1 in development and in vascular homeostasis, it is not surprising that its expression and activity are tightly regulated at the transcriptional level. The human ET-1 gene has a TATA box-containing promoter, and several cis-acting elements have been implicated in transcriptional regulation of ET-1 mRNA. An upstream activator protein-1 (AP-1) site located at position −117, which is bound by c-fos and c-jun, is required for inducible high-level ET-1 promoter activity (9). In addition, a GATA motif at position −135, which is bound by the zinc finger transcription factor GATA-2 in endothelial cells (10), is crucial to the basal and

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1 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.
regulated expression of the ET-1 gene. Furthermore, the cooperativity 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/DB1) (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 through 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. The plasmid pCMV-βGal (Stratagene) contains the β-galactosidase gene driven by the CMV promoter.

Reporter constructs containing fragments of the hET-1 5′-flanking region were inserted into pGL2-Basic and named according to the length of the fragment (from the transcriptional start site) in the 5′ and 3′ directions. For example, plasmid pGL2−204/+170 contains a hET-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, pGL2−115/+170, and pGL2−42/+170 were created by cloning the EcoRI/BglII fragment of plasmids p-204CAT, p-143CAT, p-129CAT, p-5CAT, and p-42CAT, respectively (16), in the appropriate orientation upstream of the luciferase gene of plasmid pGL2-Basic. These constructs share a common 5′ BglII site but differ at the 5′ end located at base pairs −204, −143, −129, −95, 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 PouIV/NotI-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 (NIAID, National Institutes of Health, Bethesda, MD) and has been described elsewhere (18).

Mutagenesis—Site-directed mutagenesis of the hET-1 promoter was performed by polynucleotide chain termination method (19) 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 TTACCCCCACTC was mutated to TTACATCCACTC using the mismatched primers 5′-GTCAGAGCTGTTTACATCCACTCCTATTAGGG-GTTC-3′ and 5′-GAACCCCTATAGGTTAAGACGCTTCGAC-3′. The sequence of the mutated polymerase chain reaction fragment was confirmed by the dideoxy chain termination method.

Cell Culture—The mouse myocardiudial 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 1.0 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-Promoter, pGL2-Basic, or pGL2-KDR/flk1 and the indicated concentration of pME18S-DB1 and/or p40TAX plasmid using the LipofectAMINE method (Life Technologies). pME18S was used to normalize the total concentration of plasmid. To correct for variability in transfection efficiency, 0.25 μg of pCMV-βGal was co-transfected in all experiments. 48 h after transfection, cells were lysed with 150 μl of 1× reporter lysis buffer (Promega) according to the manufacturer’s recommendation, and 50 μl of cell extracts were used for the luciferase assay. 30 μl of cell extract were used for the β-galactosidase assay as previously described (17). The ratio of luciferase activity to β-galactosidase activity in each sample served as a measure of the normalized luciferase activity. The normalized activity was expressed as fold-induction. Each construct was transfected at least 4 times, and data for each construct are presented as the mean ± S.E.

Recombinant Protein Production—A Vezf1/DB1 glutathione S-transferase (GST) fusion protein was created by inserting the Vezf1/DB1 cDNA in-frame in vector pGEX3 (Amersham Pharmacia Biotech) to create plasmid pGEX-VEZF/DB1. Recombinant protein was expressed in BL21Gold(DE3) bacterial cells (Stratagene). Cells were grown at 37 °C to A590 0.4, then isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 1 mM. 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 Coomassie Blue staining.

Intracellular Localization—Full-length Vezf1/DB1 cDNA was inserted into the green fluorescent protein (GFP) fusion vector pEGFP-C3 (CLONTECH). GFP-CHIP has been previously described (20), and expresses a GFP fusion with carboxyl terminus of Hsp70-interacting protein (CHIP). Plasmid pEGFP-C3, expressing GFP alone, served as a control for nonlocalized expression. Plasmids (1 μg) were transfected into MEC by the LipofectAMINE method. After 24 h, cells were examined for GFP expression by direct epifluorescence using a Nikon Diaphot 300 microscope.

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 washed with cold phosphate-buffered saline, then 1 ml of cold phosphate-buffered saline was added to each plate, scraped with a rubber policeman, and the cell content collected in 1.5-ml Eppendorf tubes on ice and centrifuged at 5000 rpm for 30 s at 4 °C. The supernatant was aspirated and cells were resuspended in 800 μl of hypertonic buffer (10 mM HEPES, pH 7.9, at 4 °C, 1.5 mM MgCl2, 10 mM KCl). Cells were centrifuged at 5000 rpm for 30 s at 4 °C, the supernatant was discarded and cells were resuspended in 800 μl of hypertonic buffer and
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.

Antisera—Polyclonal antisera were generated in rabbits against a peptide (MEANWTFALFPGAHEC) corresponding to a conserved region of Vezf1/DB1 coupled to keyhole limpet hemagglutinin 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. 6A. 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′-CCACCT-GGTGCCAGTC-3′) as a nonspecific competitor. To characterize specific DNA-binding proteins, we incubated nuclear extracts with affinity purified rabbit polyclonal anti-Vezf1/DB1 antibody or a similarly prepared antibody to the unrelated sequence QEKLISEENL, 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 genes. As the 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 GFP-Vezf1/DB1 fusion by epifluorescence with nuclear localization; upper left, the same field analyzed by light microscopy; middle right, expression of GFP-CHIP demonstrating cytoplasmic localization; lower right, GFP alone demonstrating both nuclear and cytoplasmic localization. Original magnification ×100.

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 hET-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 p40Tax 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 recapitulated 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. 6). This experiment shows that recombinant Vezf1/DB1 can, indeed, form a DNA-protein complex with the 3′/H11032 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 transactivate 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 (MEANWTAFLFQAHEC). 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-}

mation of specific DNA-protein complex A was attenuated, and a supershifted band (SS) appeared, when nuclear extracts were preincubated with the specific antibody, whereas a similarly prepared nonspecific antibody did not have any effect on the binding pattern (Fig. 8A).

The supershift experiment strongly suggested that Vezf1/DB1 was present in MEC nuclear extracts and participated in nuclear protein complex formation with the human endothelin-1 promoter. As additional confirmation that Vezf1/DB1 bound specifically to the ET-1 5'-flanking sequence, we examined the interaction of purified Vezf1/DB1 to the ET-1 primer (Fig. 8B). Under the conditions employed, a stable complex could form when recombinant GST-Vezf1/DB1 (but not GST alone) was used in these binding assays. As before, these complexes could be disrupted by an excess of specific cold oligonucleotide, but not by a similar concentration of a nonspecific competitor. This complex could be supershifted with an anti-GST antibody but not by a nonspecific antibody, indicating that these complexes were formed with the fusion protein, and not by a co-purifying activity. In addition, these complexes could also be abolished by the specific anti-Vezf1/DB1 antibody (data not shown). These experiments demonstrate that nuclear extracts from MEC contain Vezf1/DB1 protein and that Vezf1/DB1 is involved in a specific DNA-protein complex with the hET-1 5'-flanking sequence that contains the Vezf1/DB1 response element.

Vezf1/DB1 Trans-activates the hET-1 Promoter Maximally in Endothelial Cells—Because the expression of Vezf1/DB1 is restricted to the vascular endothelium (14), we wanted to determine whether its forced expression was sufficient to trans-activate the ET-1 promoter in non-endothelial cell types. To test this, we transiently transfected MEC or non-endothelial cell lines (C2C12 and NIH/3T3) with either the wild-type (A) or mutated hET-1 promoter constructs and the Vezf1/DB1 expression plasmid. Consistent with our previous results, Vezf1/DB1 potently trans-activated the hET-1 promoter by over 20-fold, whereas a 2-bp mutation in the Vezf1/DB1 response element abolished Vezf1/DB1 responsiveness in MEC (Fig. 9). The hET-1 promoter was also trans-activated by Vezf1/DB1 in C2C12 and NIH/3T3 cells; however, the trans-activation was only 4- and 5-fold in C2C12 and NIH/3T3 cells, respectively. This trans-activation was dependent on the same Vezf1/DB1 response element that is functional in MEC (compare wild-type and mutated promoters). These results indicate that Vezf1/DB1 is sufficient to trans-activate the hET-1 promoter in both endothelial and non-endothelial cells when it is expressed ectopically. It is likely that the differences in trans-activation among cell types reflect the presence of additional factors in endothelial cells that are required for maximal activation of the hET-1 promoter by Vezf1/DB1. This interpretation is consistent with the presence of multiple DNA-protein complexes seen in our EMSA experiments (Figs. 5, 6, and 8).
**DISCUSSION**

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 data base. 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 Cysh/His 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 lie upstream of

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2 J. Aitsebaomo and C. Patterson, unpublished observations.
Vezf1/DB1 and Endothelin-1 Expression

**Fig. 9.** Vezf1/DB1 transactivation of the hET-1 promoter in endothelial and non-endothelial cells. The luciferase reporter construct pGL2–204/170, either with a wild-type (WT) or mutated (MUT) Vezf1/DB1 response element, was transiently transfected into MEC, NIH/3T3, or C2C12 cells with the indicated concentrations of the Vezf1/DB1 expression vector. The vector pCMV-βGal was used to correct for differences in transfection efficiency. The results are expressed as the mean ± S.E.

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-12 (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, endothelial cells during vascular development, then absence of Vezf1/DB1 in mice should phenocopy, at least partially, endothelial cells during vascular 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 endothelium-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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