Octamer-dependent in Vivo Expression of the Endothelial Cell-specific TIE2 Gene*

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The TIE2 gene, also known as TEK, encodes a tyrosine kinase receptor that is required for the normal development of the vascular system during embryogenesis. TIE2 is specifically expressed in endothelial cells; however, the transcriptional mechanisms that regulate this highly restricted pattern of expression remain unknown. Here we demonstrate that a consensus octamer element located in the 5’-flanking region of TIE2 is required for normal expression in embryonic endothelial cells. Transgenic embryos carrying a TIE2/LacZ construct spanning 2.1 kilobases of upstream regulatory sequences exhibit expression of the reporter transgene specifically in endothelial cells. Site-directed mutagenesis of a consensus octamer element located in this region results in the loss of enhancer activity and significantly impairs the endothelial expression of the reporter transgene. Consistent with the in vivo data, in vitro DNA-protein binding studies show that the consensus octamer element displays an endothelial cell-specific pattern of binding, suggesting an interaction with a protein complex consisting of Oct1 and an endothelial cell-restricted cofactor. These data identify a novel role for the octamer element as an essential regulator of TIE2 expression, define the first known transcriptional pathway that mediates the expression of a developmental endothelial cell gene, and provide insights into the transcriptional mechanisms that regulate development of the vasculature during embryogenesis.

Studies in vertebrates indicate that the vasculature is the first organ system to develop and to function during embryogenesis and that formation of blood vessels is required for the normal development of other organ systems. In the adult organism, formation of de novo blood vessels is required in several physiological conditions including wound healing and regeneration of the female reproductive system and in pathological conditions such as the growth of solid tumors and metastases (1).

Embryological studies indicate that blood vessels develop through two distinct mechanisms, vasculogenesis and angiogenesis. Vasculogenesis consists of the in situ differentiation of endothelial cells from mesoderm-derived precursors termed angioblasts, with the subsequent assembly of differentiated cells into blood vessels (2). Angiogenesis represents the formation of new blood vessels through sprouting and branching of endothelial cells from pre-existing vessels (3). The expression of specific molecules on the surface of endothelial cells allows these cells to regulate the vascular developmental process through interaction with ligands produced by periendothelial mesenchymal cells. VEGF-R2 (flk1), the first of these receptors to be expressed, binds vascular endothelial growth factor and is required for the earliest stages of endothelial cell differentiation to initiate the process of vasculogenesis (4, 5). VEGF-R1 (flt1), also a receptor for vascular endothelial growth factor, plays a role in later stages of vasculogenesis including the assembly of differentiated endothelial cells into vascular channels (6, 7). TIE1, an orphan receptor, is essential for the maintenance of vascular integrity once blood vessels have formed (8). TIE2, a receptor for angiopoietin-1 and angiopoietin-2, plays a more complex developmental role (9). It is required for the angiogenic process, the remodeling of the primary vascular plexus into large and small vessels, the development of the endocardial layer, and the recruitment of periendothelial cells to the vascular wall (8, 10).

Although significant progress has been made in determining the role of endothelial cell surface molecules in the process of vascular development, very little is known of the transcriptional pathways that regulate the expression of these molecules and thus coordinate fundamental events leading to blood vessel formation. To elucidate the mechanisms that regulate the expression of endothelial developmental genes, we took advantage of in vivo transcription studies conducted on the TIE2 promoter in transgenic mice. These studies have previously shown that an upstream region encompassing 1.2 kb of the murine TIE2 gene is sufficient to confer highly specific reporter gene expression in embryonic endothelial cells and that deletion of an internal 223-bp region abolishes this cell-specific enhancer activity (11). These data indicate the presence of important cis-acting regulatory elements within the 223-bp enhancer region, being required for the cell-specific expression of TIE2 in embryonic endothelial cells. In this report, we demonstrate that a consensus octamer element (ATTTGCAT) contained within the 223-bp region is required for in vivo cell-specific enhancer activity in endothelial cells. DNA-protein binding studies indicate that the octamer element exhibits an

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1 The abbreviations used are: kb, kilobase(s); EMSA, electrophoretic mobility shift assay; BAEC, bovine aortic endothelial cells; YSC, yolk sac cells; bp, base pair(s); Eν, embryonic day n; X-gal, 5-bromo-4-chloro-3-indolyl β-d-galactopyranoside; FBS, fetal bovine serum.

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endothelial cell-specific pattern of binding and that the cell-specific complex consists of the ubiquitous factor Oct1 and an endothelial cell-specific cofactor that is distinct from the B-cell cofactor Bob1/OCa-B/0BF-1. Our data suggest that the interaction of the Oct1-cofactor complex with the consensus octamer element is likely to mediate the expression of the TIE2 gene in endothelial cells. These results show a central role for the octamer element in regulating TIE2 expression and in mediating the development of the vascular system during embryogenesis.

**EXPERIMENTAL PROCEDURES**

**DNA Sequencing of the 223-bp Region of TIE2**—We have previously reported on the cloning of the promoter and 5′-flanking region of the murine TIE2 gene (12). For DNA sequencing, the 223-bp SacI-SphI region was subcloned into a pGLO2 basic vector, and dieodeoxy chain termination sequencing was performed using Sequenase 2.0 according to the manufacturer’s instructions (U. S. Biochemical Corp.).

**Reporter Gene Constructs**—Three separate DNA constructs were used to generate transgenic mice, TIE2.wt.LacZ, TIE2.2Octmut.LacZ, and TIE2.1Octmut.LacZ. The TIE2.wt.LacZ construct consists of 2.1 kb of murine TIE2 regulatory sequence (which encompasses the 223-bp region, 0.8 kb of 5′ flanking sequence and 318 bp of 5′-untranslated region) subcloned upstream of the LacZ gene containing a nuclear localization sequence. The TIE2.2Octmut.LacZ vector is identical to the TIE2.wt.LacZ except for point mutations introduced in both consens and atypical octamer sites (ATTGTGATT → GTGGCATG and ATGCTAAT → ATGCTAATC). The TIE2.1Octmut.LacZ vector contains point mutations only in the consensus octamer site (ATTGTGATT → GTGGCATG).

The TIE2.wt.LacZ was generated by subcloning the LacZ gene from PPP46.21 containing a nuclear localization signal into the HindIII-NolI sites of pKS-Bluescript (Stratagene). The 2.1-kb HindIII-HindIII fragment of TIE2 was then subcloned in the same vector to generate pKS.TIE2.wt.LacZ. Digestion of this vector with Sall and NolI liberated the transgene cassette TIE2.wt.LacZ. Site-directed mutagenesis of the octamer elements was introduced using the Altered Sites II in vitro mutagenesis kit (Promega) according to the manufacturer’s instructions to generate TIE2.2Octmut.LacZ and TIE2.1Octmut.LacZ. The mutations were verified by DNA sequencing.

**Production of Transgenic Mice, Genotyping Embryos, and β-Galactosidase Staining**—The TIE2.wt.LacZ, TIE2.2Octmut.LacZ, and TIE2.1 Octmut.LacZ constructs were purified from the plasmid vector, dephosphorylated, and then end-labeled with [γ-32P]ATP (NEN Life Science Products). For EMSA, binding conditions consisted of 40 μl NaCl, 27 mM KCl, 10 mM Hepes, pH 7.9, 0.5 mM EDTA, 0.75 mM dithiothreitol, 4% (v/v) Ficoll, 0.2% (v/v) glycerol, 20 ng/ml herring sperm DNA (16). 6 μg of nuclear extracts were incubated at 20°C for 30 min and then the binding of the labeled oligos and, when indicated, 100-fold excess of unlabeled oligos were added, and the reactions were incubated at room temperature for an additional 20 min. For supershift assays, 3 μg of rabbit polyclonal anti-Oct1 antibody, anti-Bob1 antibody (Santa Cruz Biotechnology), or rabbit IgG were preincubated with nuclear extracts for 30 min at room temperature using the same conditions described above prior to the addition of labeled oligonucleotides. Samples were then loaded onto a 5% polyacrylamide gel and electrophoresed in 1 × Trisglycine buffer at 200 V for 5 h at 4°C.

**Northern Blot Analysis**—Poly(A+) RNA from BAEC, YSC, HeLa, Namalwa, and erythroleukemia cells were obtained using the MicroFastTrack (Invitrogen) according to the manufacturer’s recommendations. The RNAs were electrophoresed on a formaldehyde–7% polyacrylamide gel, transferred to a Magna Charge membrane (Micron Separations, Inc.), baked for 2 h at 80°C, and linked using a UV cross-linker (Stratagene). The membrane was then prehybridized for 4 h at 37°C, hybridized with a full-length human Bob1 cDNA probe (generously provided by Drs. Walter Schaffner and Oleg Georgiev, University of Zurich, Switzerland), and labeled using the Primer-it Random Primer Labeling kit (Stratagene). After washing twice at room temperature and once at 37°C under low stringency conditions (2 × SSC, 0.5% SDS), the membrane was subjected overnight to autoradiography at 80°C. The membrane was then stripped and rehybridized with a human cyclin C1 DNA probe.

**RESULTS**

**The Upstream Regulatory Region of TIE2 Contains Two Putative Sites for Octamer Binding Proteins**—Experiments employing reporter transgenes in mice have indicated that a 223-bp regulatory region located between −760 and −537 bp is essential for high level specific expression of the murine TIE2 gene in endothelial cells of the mouse embryo (11). To guide studies aimed at investigating the cis-acting DNA elements that mediate cell-specific enhancer activity, we first evaluated this minimal region for the presence of known DNA binding sites. DNA sequence analysis revealed the presence of three putative binding sites for transcription factors: a consensus octamer element 5′-ATTGGCAT-3′ (complementary sequence being 5′-ATGCAAAT-3′ at −669 bp, a CCAT motif at −653 bp, and an atypical octamer element 5′-ATGCTAAT-3′ at −574 bp (Fig. 1). The atypical octamer element differs from the consensus sequence by the presence of a T instead of an A in the fifth position, and both octamer elements are known to bind members of the POU domain family of transcription factors (17). Several of these factors regulate important developmental events in the vertebrate embryo. Examples include the terminal differentiation of B-cells mediated by Oct2, the development of the hypothalamus mediated by Brm2 and the determination of the somatotrope, lactotrope, and thyrotrope lineages.
in the pituitary gland by Pit-1 (18). Therefore we investigated whether the octamer elements contained in the TIE2 enhancer region are required for the expression of this vascular developmental gene in embryonic endothelial cells.

The Consensus Octamer Element Contained in the 5′-Flanking Region of TIE2 Is Required for Specific Enhancer Activity in Embryonic Endothelial Cells—To determine whether the octamer elements may function as endothelial cell enhancers for TIE2, we evaluated the expression of LacZ in transgenic mice under the control of a wild type or mutant TIE2 enhancer region (Fig. 2). The wild type construct (TIE2 wt LacZ) consisted of the LacZ gene containing a nuclear localization signal cloned downstream of the 2.1-kb TIE2 sequence. The double mutant construct (TIE2 2Octmut LacZ) is identical to the wild type construct except that point mutations were introduced in both octamer elements to prevent interaction with octamer binding proteins. The TIE2 regulatory region used to generate these constructs differs from the one used by Schlaeger et al. (11). Both sequences have a common 3′ end at a HindIII site located at +318 bp; however, our construct extends further upstream to a HindIII site located at −1.8 kb instead of −0.9 kb. Because the TIE2 enhancer mediates reporter transgene expression only in embryonic endothelial cells and because native TIE2 expression begins at approximately E8.0, we analyzed LacZ expression in embryos at E9.5 as previously done (11, 19).

Of the first generation embryos carrying the wild type construct, 38% did incorporate the transgene as demonstrated by polymerase chain reaction of yolk sac tissue using LacZ-specific primers. Of these transgenic embryos, 44% demonstrated strong LacZ staining that is restricted to endothelial cells (Table I). Endothelial cells of the dorsal aorta, branchial arch arteries, intersomitic arteries, vitelline artery, cardinal veins, sinus venosus, the endocardium of the common atrium and ventricle, outflow tract, and yolk sac stained positively (Fig. 3). Although the intensity and extent of LacZ expression varied slightly among the transgenic embryos, all those that stained demonstrated strong endothelial expression, and none showed evidence of ectopic nonendothelial staining. Overall, most embryonic endothelial cells stained positively for LacZ, and these findings agree with the study reported by Schlaeger et al. (11). As expected, LacZ expression in endothelial cells was not uniform, and expression was also weaker and more patchy in the ventricular endocardium as compared with the aorta and atrial endocardium. Taken together, these data indicate that the 2.1-kb regulatory region of TIE2 contains DNA elements that can provide cell-specific expression of the TIE2 promoter in embryonic endothelial cells.

Transgenic embryos carrying the double mutant construct (TIE2 2Octmut LacZ) were collected and analyzed in a manner identical to the wild type construct embryos. A similar percentage of these embryos demonstrated evidence of LacZ staining (42%) as compared with the wild type embryos (Table I). However, the pattern and intensity of LacZ expression in the embryos carrying the double mutant construct markedly differed from those with the wild type construct. In the embryos that showed evidence of β-galactosidase expression, staining was patchy and weak and mostly limited to few endothelial cells in the entire embryo (Fig. 4). In particular, endothelial cells in the aorta, intersomitic arteries, and atrial endocardium that showed strong staining with the wild type construct did not stain or showed weak staining limited to a few cells. Of interest was the absence of a particular pattern of LacZ expression in these embryos. Some embryos showed LacZ staining in a few endothelial cells within the aorta or in the intersomitic arteries; others showed staining only in the yolk sac or in the cardinal veins (Fig. 4). Of 11 mutant construct embryos that stained positively for LacZ, a single embryo demonstrated strong staining in the aorta and endocardium; however, the number of stained endothelial cells was significantly less than that for the wild type construct embryos. These data show that mutation of the octamer elements markedly impairs reporter gene expression and ablates most of the enhancer activity in endothelial cells.

Because the consensus octamer element is known to mediate cell-specific enhancer activity in other cell lineages such as B lymphocytes, we examined whether this element, rather than the atypical octamer motif mediates endothelial cell enhancer activity of TIE2. Transgenic embryos carrying point mutations only in this element (TIE2 1Octmut LacZ) were examined as described above. These embryos exhibited LacZ expression that is strikingly similar to the pattern seen with the double mutant construct (Fig. 5). In particular, none of the embryos demonstrated any significant endothelial cell staining, and LacZ expression was uniformly very weak and patchy, mostly limited to few endothelial cells in the whole embryo. Similar to the
embryos carrying the double mutant construct, there was no particular pattern of LacZ staining, with all the stained embryos showing weak and very limited staining in the intersomitic arteries or the aorta. These findings indicate that the consensus octamer element is required for normal expression of TIE2 in embryonic endothelial cells and is the DNA element that mediates most if not all of the endothelial cell enhancer activity of the TIE2 promoter.

The Consensus Octamer Element Mediates Protein Binding of the 223-bp Enhancer Region—To investigate the mechanisms by which the consensus octamer element mediates endothelial cell-specific expression and to obtain a correlation with the in vivo functional studies, we conducted a series of DNA-protein interaction experiments. As a first step, a DNA fragment spanning the entire 223-bp enhancer region was radiolabeled and employed in standard EMSA (16). Nuclear extracts were prepared from a cultured YSC line derived from the murine yolk sac tissue at approximately 8.5 days of embryonic development (15). These cells serve as a model of embryonic endothelial cells because they express endothelial markers.
such as flk1 and TIE2 and are able to form microvascular-like tubular structures in culture (20, 21). EMSA employing extracts from these cells revealed a single retarded complex, indicating that the 223-bp DNA sequence is capable of binding protein (Fig. 6). The retarded complex was competed when excess unlabeled DNA was included in the reaction, indicating specificity of the DNA-protein interaction. Unlabeled oligonucleotides encoding the consensus octamer element were able to fully compete for binding to the DNA fragment, whereas those encoding the atypical octamer element were not. The data suggest that a protein binding sequence is contained within the 223-bp region of the TIE2 gene and that this sequence is the consensus octamer element. These findings are in agreement with our in vivo data and suggest a mechanism for endothelial cell-specific expression that is mediated by the consensus octamer element.

**The Consensus Octamer Element Binds Nuclear Protein(s) in an Endothelial Cell-specific Manner**—Because the consensus octamer was able to compete for protein binding to the 223-bp region, we investigated its pattern of binding to a number of endothelial and nonendothelial cell extracts. For this purpose, EMSA were employed using a radiolabeled oligonucleotide probe spanning the octamer element in conjunction with nuclear extracts derived from three endothelial and three nonendothelial cell types. Endothelial cells included YSC, BAEC, and murine hemangioendothelioma cells. Nonendothelial cells included Namalwa B-cells, HeLa cells, and myoblasts. The three endothelial cell types showed two specific retarded bands, complex A and B, whereas the three nonendothelial cell types showed only one of the retarded bands, complex A (Fig. 7). This suggests that complex B, the more slowly migrating of the two bands, is likely to result from the specific interaction between the octamer element and protein factor(s) present exclusively in endothelial cell extracts. These findings correlate with our in vivo data and suggest a mechanism for endothelial cell-specific expression that is mediated by the consensus octamer element.

**The Endothelial Cell-specific Band Consists of a Protein Complex That Contains Oct1 and Does Not Result from Binding of an Oct1-Bob1 Complex to the Octamer Element**—A series of experiments were conducted to characterize the nature of the
endothelial cell-specific band noted on EMSA. For this purpose, EMSAs were performed using nuclear extracts derived from BAEC and Namalwa B-cells and a labeled oligo spanning the consensus octamer element in the presence of anti-Oct1 antibodies (Fig. 8). Because complex A is seen with all the cell lines used in EMSA, we hypothesized that it may represent the binding of the ubiquitously expressed transcription factor Oct1 to the octamer element. This was confirmed by the addition of anti-Oct1 antibodies, which resulted in a supershift of complex A in both BAEC and Namalwa cells (lanes 2 and 6). Furthermore, the addition of anti-Oct1 antibodies but not control IgG also resulted in the supershift of the endothelial cell-specific band, complex B (lane 2). Because the anti-Oct1 antibodies recognize a 20-amino acid epitope that is specific to Oct1 and not shared by other known members of the POU domain family of transcription factors (22–25), these findings indicate that complex B contains Oct1. The addition of control IgG did not affect either complex A or complex B (lane 4).

Oct1 is well known to make protein-protein interactions with a number of transcription factors. Most of these factors including Sp-1, Ap-1, PTF, and the steroid receptors have a wide pattern of expression (26–29), whereas others such as VP16 and the adenovirus DNA polymerase are viral proteins (30, 31). One factor in particular, Bob1, also known as OCA-B and
OFB-1, has a highly restricted pattern of expression, being constitutively expressed in B lymphocytes (32–34). Targeted mutagenesis studies indicate that Bob1 is required for antigen-induced expression of immunoglobulins (35, 36). Because endothelial and hematopoietic cells are derived from a common progenitor cell, the hemangiblast (37), we were compelled to determine whether the endothelial cell-specific complex B represents the interaction of an Oct1-Bob1 protein complex with the octamer element, especially because the expression of Bob1 has not been previously evaluated in endothelial cells. The addition of anti-Bob1 antibodies to the EMSA reaction did not affect either of complex A or B in BAEC and Namalwa cells, indicating that this ubiquitous complex represents the binding of the Oct1 protein to the octamer element. Furthermore, anti-Oct1 antibodies but not anti-Bob1 antibodies or control IgG obliterate complex B in endothelial cells (lanes 2–4), indicating that this complex is likely to result from the binding of Oct1 and an endothelial cell cofactor to the octamer element. Anti-Bob1 antibodies but not control IgG result in the supershift of the Oct1-Bob1 complex in Namalwa cells (lanes 7 and 8).

**DISCUSSION**

Despite the large number of endothelial cell genes studied to date, the molecular mechanisms that mediate specific gene expression in this cell type remain obscure. Most transcription studies have been based on in vitro DNA-protein binding experiments and, so far, have not allowed the identification of cell-specific DNA elements or transcription factors. The application of transgenic mice technology to the study of transcriptional regulation has facilitated this task by allowing the identification, within large sequences of promoters and enhancers, of minimal regions capable of conferring cell-specific expression of the gene of interest.
complex involving Oct1, the cellular protein HCF (host cell factor) and the viral regulatory factor VP16 (43, 44). Both consensus and atypical octamer elements can mediate cell-specific gene expression. The Oct1-Bob1 complex requires an A in position 5 for binding and thus interacts only with the consensus element (17), whereas the Oct1-HCF-VP16 complex requires a T in position 5 and thus interacts only with the atypical element (30).

In addition to TIE2, the DNA regulatory regions of three other endothelial cell genes have been studied in transgenic mice. These include endothelin-1, von Willebrand factor, and TIE1 (45–47). The murine endothelin-1 DNA construct used in the reported study conferred adequate levels of reporter gene expression in the endothelial cells of adult mice; however, expression was not restricted to the endothelium because this was also evident in epithelial and mesangial cells (46). In the study using the human von Willebrand factor promoter, expression of the reporter transgene was restricted to a small number of endothelial cells in the brain and the yolk sac (45). Taken together, these findings indicate that the regulatory regions of endothelin-1 and von Willebrand factor under study do not contain all the DNA elements required for cell-specific expression throughout the endothelium. In contrast, a 900-bp regulatory region of the murine TIE1 gene allows high level expression of the reporter transgene specifically in embryonic endothelial cells (47). Several similarities exist in the results of the TIE2 and TIE1 reporter transgene experiments. The pattern of expression of the reporter transgenes is similar, endothelial cell expression does not fully reproduce the pattern of the endogenous genes, and expression is markedly down-regulated following birth. Analysis of the DNA sequence of the 900-bp TIE1 regulatory region reveals the presence of a consensus octamer element located at -466 bp (47). Because TIE1 and TIE2 belong to the same family of tyrosine kinase receptors, both are initially expressed in endothelial cells at approximately E8.0–E8.5, and both serve a similar developmental function, the possibility exists that both factors share a common mechanism of gene expression mediated by the octamer element.

Expression of the reporter transgene in embryonic endothelial cells under the control of the upstream TIE2 enhancer is not uniform and does not fully reproduce the pattern of the native TIE2 gene. This emphasizes the heterogeneity and diversity of endothelial cells of the various vascular beds in regard to their developmental origin and the mechanism of vascular formation. Furthermore, reporter gene expression is markedly down-regulated following birth, suggesting a switch in the mechanisms that regulate TIE2 expression at different stages of development (11). These findings indicate a requirement for additional DNA elements located outside the upstream regulatory region. Recent studies show that a DNA construct containing a 303-bp intronic element in addition to the upstream regulatory region provides a uniform expression to virtually all endothelial cells of the embryo and adult mouse (38).

Data from this study provide new findings regarding the molecular mechanisms that mediate the formation of the vascular system during embryogenesis and identify the octamer element as the first essential transcriptional regulator of this developmental process. We have yet to determine whether the octamer element is sufficient for cell-specific enhancer activity and whether cell specificity results from the binding of a single transcription factor or of a larger multiprotein complex. The identification and characterization of such transcription factor(s) should provide an early marker of the endothelial lineage and should lead to a better understanding of the molecular mechanisms that govern endothelial cell differentiation and the process of vascular formation during embryogenesis.

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