Uniform vascular-endothelial-cell-specific gene expression in both embryonic and adult transgenic mice

(transcription/enhancer)

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ABSTRACT TIE2 is a vascular endothelial-specific receptor tyrosine kinase essential for the regulation of vascular network formation and remodeling. Previously, we have shown that the 1.2-kb 5'-flanking region of the TIE2 promoter is capable of directing β-galactosidase reporter gene expression specifically into a subset of endothelial cells (ECs) of transgenic mouse embryos. However, transgene activity was restricted to early embryonic stages and not detectable in adult mice. Herein we describe the identification and characterization of an autonomous endothelial-specific enhancer in the first intron of the mouse TIE2 gene. Furthermore, combination of the TIE2 promoter with an intron fragment containing this enhancer allows it to target reporter gene expression specifically and uniformly to virtually all vascular ECs throughout embryogenesis and adulthood. To our knowledge, this is the first time that an in vivo expression system has been assembled by which heterologous genes can be targeted exclusively to the ECs of the entire vasculature. This should be a valuable tool to address the function of genes during physiological and pathological processes of vascular ECs in vivo. Furthermore, we were able to identify a short region critical for enhancer function in vivo that contains putative binding sites for Ets-like transcription factors. This should, therefore, allow us to determine the molecular mechanisms underlying the vascular-EC-specific expression of the TIE2 gene.

Establishment of the circulation is critical for the development of all organ systems of the human body, and endothelial cells (ECs), which line the lumina of all blood vessels, play an essential role in the formation and physiological functions of the circulatory system (for reviews, see refs. 1 and 2). In addition to the 2.1-kb promoter, pHHNS contains a mouse TIE2 genomic fragment extending from an end of exon 1 approximately 10 kb into the first intron of the mouse TIE2 gene.

MATERIALS AND METHODS

Reporter Gene Construct. LacZ reporter constructs were based on pBSIHSK(+) or pBSISK(+) (Stratagene). pHH—consisted of the murine 2.1-kb HindIII TIE2 promoter fragment followed by the LacZ reporter gene and simian virus 40 poly(A) signal sequence from pβ-actinPSDKLacZpA (a gift from Janet Rossant, Mount Sinai Research Institute, Toronto, Canada). In addition to the 2.1-kb promoter, pHHNS contains a mouse TIE2 genomic fragment extending from an NgoMI site at the 3' end of exon 1 approximately 10 kb into the first intron downstream of the LacZ cassette.

Abbreviations: EC, endothelial cell; BAEC, bovine aortic EC; E, embryonic day; bZIP, basic domain-leucine zipper; NF-S, nuclear factor S. Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. U85629).

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Deletions of pHHNS from the 3' end of the first intron fragment yielded pHHNI, pHHNb, pHHRN, and pHHRNK, respectively (see Fig. 3 for details). pHHIX contained the XhoI–KpnI enhancer fragment downstream of the reporter gene. Systematic 3' deletions of this XhoI–KpnI fragment yielded pHHIX, pHHIXB, pHHIXN, and pHHIXX, respectively (see Fig. 3 for details). For construction of ptk–, the TIE2 promoter of pHH– was replaced with the herpes simplex virus 1 thymidine kinase gene (tk) minimal promoter (nucleotides −116 to +68) from PMCIPOLA (Stratagene). ptkXX and pKXK contained the XhoI–KpnI enhancer fragment in sense orientation downstream of the reporter gene and in antisense orientation upstream of the tk promoter, respectively. ptkNcXb contained the NcoI–XbaI intron fragment downstream of the tk-promoter driven LacZ. ptkXXΔ was identical to ptkXX, except that it lacked the SacI–XbaI region from the NcoI–XbaI intron fragment. pHHXK-SaXb was identical to ptKXKΔ, except that it contained the 2.1-kb TIE2 promoter instead of tk promoter. Site-directed mutagenesis of the XhoI–KpnI enhancer was performed with the Chameleon kit (Stratagene). The sequence of all constructs was confirmed.

Cell Culture and Transfection Analysis. Primary bovine aortic EC (BAEC) cultures were kept as described (15). The cells were split 1:5 into 35-mm dishes and transfected 24 h later with 5 μg of DNA [0.5 μg of simian virus 40 enhancer/promoter-driven luciferase plasmid, the respective construct at 0.25 μg/kb, and pBSIKS(+)]) with 10 μl of Lipofectin (GIBCO/BRL). Human embryonic kidney cells (HEK293 cells, American Type Culture Collection catalogue no. 1573) were split 1:3 into 12-well plates and transfected 24 h later with a total of 360 ng of the same DNA mixtures (used for the BAEC transfections) using the MBS transfection kit (Promega), and a MicroLumat LB96P luminometer (Berthold, Nashua, NH). Background values obtained with lysates from mock-transfections were subtracted, and the β-galactosidase activity of each extract was normalized to the luciferase activity of each extract. The mean from at least six values from at least three experiments.

Transgene Preparation and Transgenic Mice. Preparation of DNA (lacking any plasmid backbone sequences) for oocyte injection, microinjection, surgical procedures, and genotyping were performed as described (4). However, to genotype transgenic mice carrying tk core-promoter constructs, the upstream

![HH-](image1.png) ![HHNS](image2.png)

**Fig. 1.** Whole-mount 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside-stained embryos at E11.5. (a) An embryo transgenic for the construct HH– shows the reduced staining, as described (4). (b) An embryo transgenic for the construct HHNS shows specific staining in virtually all vessels. TG, number of transgenic embryos analyzed; ES, number showing the endothelial-specific staining shown in the respective picture; ET, number showing ectopic staining; NO, number showing no staining at all.
KpnI digesting that certain elements lying outside of this 1.7-kb fragment (data not shown). Moreover, certain vascular beds such as kidney showed a less uniform (but still EC-specific) expression pattern in vivo. However, in adult animals the two independently established HHXK transgenic lines (183.2 and 183.11) showed a less uniform (but still EC-specific) expression pattern (data not shown). Moreover, certain vascular beds such as kidney glomeruli were devoid of LacZ staining (data not shown), suggesting that certain elements lying outside of this 1.7-kb XhoI–KpnI region but within the 10-kb fragment are essential to maximize the enhancer activity in adult tissues.

Table 1. Summary of the in vivo activity of the enhancer

<table>
<thead>
<tr>
<th>Construct</th>
<th>TG</th>
<th>ES</th>
<th>ET</th>
<th>NO</th>
</tr>
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<tbody>
<tr>
<td>HHXK</td>
<td>14</td>
<td>9</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>tkXK</td>
<td>13</td>
<td>6</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>KXtk</td>
<td>12</td>
<td>7</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>tkNcXb</td>
<td>15</td>
<td>9</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>tk X</td>
<td>9</td>
<td>0</td>
<td>1</td>
<td>8</td>
</tr>
</tbody>
</table>

Abbreviations are as in Fig. 1. Data are presented as numbers of embryos.

To determine whether this uniform blood-vessel-EC-specific expression of the LacZ persisted in later embryonic stages and adult animals, permanent transgenic mouse lines were established with the HHXNS construct. Two transgenic lines derived from founders 182.22 and 182.30 were studied, and uniform and strong LacZ staining was detected in virtually all vessels of embryonic stage E14.5 (data not shown). In situ hybridization with a LacZ probe and immunostaining with an anti-PECAM1 antibody of adjacent sections of E14.5 embryos confirmed the EC-specific, complete, and uniform expression of the LacZ reporter (Fig. 2A–D).

In adult mice, strong and uniform LacZ staining was detected in virtually all blood vessel ECs of many tissues including brain, eye, heart, kidney, intestine, spleen (Fig. 2Ba–Bh) and other tissues (data not shown).

Identification of cis-Acting Sequences Within the First Intron That Are Essential for EC-Specific Gene Expression in Vivo. Nucleotide sequences of the first intron that are essential for EC-specific expression were identified by systematic 3′ truncation of the intron fragment. Analysis of this series of deletion constructs by transient transfection of BAECS in vitro indicated the importance of an internal 1.7-kb XhoI–KpnI fragment (Fig. 3). This sequence was found to be necessary and sufficient to enhance TIE2 promoter driven LacZ activity by approximately 50% in these cells (Fig. 3B). Transfections with 3′ deletions of the 1.7-kb enhancer revealed an internal 303-bp NcoI–XbaI fragment (NcXb) as an essential region (Fig. 3B). Transcriptional activation by the 1.7-kb XhoI–KpnI fragment appeared to be EC-specific by itself, since it was not active in human embryonic kidney cells (HEK293; see Fig. 3C). In addition, both the 1.7-kb fragment and its 303-bp subfragment were able to up-regulate the heterologous tk promoter by approximately 50% in BAECS (Fig. 3D), showing that these fragments have features of an autonomous EC-specific enhancer.

Sufficiency of these shorter intron fragments for EC-specific transcriptional activation was confirmed by transgenic studies. Transgenic mouse embryos with the construct HHXK were produced and analyzed, and they revealed a LacZ expression pattern indistinguishable from that produced with the HHXNS construct at E11.5. However, in adult animals the two independently established HHXK transgenic lines (183.2 and 183.11) showed a less uniform (but still EC-specific) expression pattern (data not shown). Moreover, certain vascular beds such as kidney glomeruli were devoid of LacZ staining (data not shown), suggesting that certain elements lying outside of this 1.7-kb XhoI–KpnI region but within the 10-kb fragment are essential to maximize the enhancer activity in adult tissues.

Table 2. Summary of the activity of different mutated constructs in transgenic embryos at E11.5

<table>
<thead>
<tr>
<th>Construct (mutated sites)</th>
<th>Wild-type sequence</th>
<th>Mutant sequence</th>
<th>TG</th>
<th>ES</th>
</tr>
</thead>
<tbody>
<tr>
<td>tkXK</td>
<td>Wild-type control</td>
<td>No mutations</td>
<td>13</td>
<td>6</td>
</tr>
<tr>
<td>tkXK (GATA+αIFN2)</td>
<td>...AAGTGATAGAAGATGAA...</td>
<td>...AAcgcgtATGAA...</td>
<td>12</td>
<td>7</td>
</tr>
<tr>
<td>tkXK (Sp1)</td>
<td>...GAGGCTGTATGAT...</td>
<td>...GAcgcgtATGAT...</td>
<td>12</td>
<td>6</td>
</tr>
<tr>
<td>tkXK (***+NS-F)</td>
<td>...CAGATCTGTACGAG...</td>
<td>...CAGAcgcgtGCC...</td>
<td>13</td>
<td>4*</td>
</tr>
<tr>
<td>tkXK (Ets1)</td>
<td>...GCTTCTCGGATG...</td>
<td>...GCacgcgt TG...</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>tkXK Δ(bZIP, CP2-γ, PEA3)</td>
<td>...GCacgcgtTG...</td>
<td>Deletion of the SacI–XhoI region</td>
<td>18</td>
<td>0</td>
</tr>
</tbody>
</table>

Abbreviations are as in Fig. 1; ***, octameric palindrome. Data are presented as numbers of embryos. *Endothelial staining was reduced or enhanced ectopic staining was observed.

To investigate whether the 1.7-kb XhoI–KpnI fragment was able to function as a bona fide EC-specific enhancer in vivo, this fragment was placed 3′ and 5′ of the LacZ cassette driven by the heterologous tk promoter, in sense (tkXK) and antisense orientation (KXtk), respectively. Strong and reproducible EC-specific LacZ expression was detected in transgenic embryos (E11.5) derived from both types of constructs (data summarized in Table 1). Furthermore, even the 303-bp NcoI–XbaI fragment (core enhancer) was able to activate the tk promoter (construct tkNcXb) in a EC-specific manner in vivo, although increased integration-site-dependent ectopic expression was observed (Table 1). None of the embryos transgenic for the LacZ reporter gene, driven by the tk promoter alone (construct tk–), showed any EC-specific staining (Table 1).

Importance of the 3′ End of the Core-Enhancer Sequence Including Putative Binding Sites for Ets1, Basic Domain-Leucine Zipper (bZIP), CP2-γ, and PEA3 Transcription Factors. Sequence analysis of the core enhancer (303-bp NcoI–XbaI fragment) revealed the presence of several putative binding sites for general, as well as tissue-specific, transcription factors and other motifs (Fig. 4 and Table 2). The sequence begins with a site for transcription factor Yin Yang 1 (YY1, or CF1). Two αIFN2 sites were found to flank a consensus site for GATA transcription factors. Further downstream, there are sites for Sp1 and nuclear factor S (NF-S), a second CF1 site, and a palindromic motif (CAGATCTG; see Discussion). At the 3′ end of the core enhancer, putative sites for transcription factors Ets1, CP2-γ, and PEA3 were identified. In addition, a palindromic motif (GAGCTC) was present that could serve as a binding site for a bZIP type of transcription factor (see Discussion).

Importance of some of these sites for the in vivo activity of the enhancer was challenged by testing constructs in which these sites were altered or deleted. Change of the putative site for αIFN2, GATA, and Sp1 did not significantly affect reporter gene expression in transgenic embryos at E11.5 (data summarized in Table 2). In contrast, the mutation destroying the octameric palindrome described above and the putative NF-S site caused a greater integration-site dependency of the corresponding construct, as fewer transgenic embryos expressed LacZ, expression was weaker and ectopic expression was observed more frequently. The mutation that changed the sequence containing the putative Ets1 site rendered the enhancer completely inactive in vivo, as did the deletion of the SacI–XbaI fragment containing the putative sites for bZIP, CP2-γ, and PEA3. As a control, the latter two mutant forms of the enhancer were also tested in combination with the TIE2 promoter. The expression pattern of these constructs resembled that of HH− (i.e., promoter alone), confirming the effect of these mutations in vivo (data not shown).

DISCUSSION

Our results show that by a combination of upstream and intronic sequences from the murine TIE2 gene, reproducible, strong, uniform, and highly specific expression of genes in virtually all ECs of transgenic embryos and adult mice can be achieved. Although we cannot exclude the possibility that some minor populations of vascular beds did not express the reporter gene, EC targeting with this expression system is by...
for the most uniform and specific compared with that mediated by other “EC-specific” promoters (5–7).

The apparent discrepancy between the down-regulation of TIE2 expression in adult vessels, as assessed by mRNA in situ hybridization studies (13), and the observed strong LacZ staining may be due to the loss of negative-regulatory elements or to the very low turnover of adult ECs, in which even a small transcriptional activity may be sufficient for the accumulation of significant amounts of a stable protein over time, such as the bacterial β-galactosidase.

Our studies of various intron fragments suggest that several transcriptional regulatory modules are present within the 10-kb intronic fragment. An internal 1.7-kb XhoI–Kpn1 fragment was identified as an autonomous EC-specific transcriptional enhancer that worked both in vitro and in vivo, in an orientation- and position-independent manner and even when combined with a heterologous promoter. However, the increased integration-site dependency of the HHXX construct (TIE2 promoter plus 1.7-kb enhancer-driven LacZ reporter) compared with the HHNS construct (TIE2 promoter plus 10-kb-fragment-driven LacZ reporter) indicates that additional regulatory sequences exist outside the XhoI–Kpn1 fragment but within the 10-kb fragment. Sequences outside the 1.7-kb enhancer are also likely to be responsible for the more uniform expression of the HHNS construct in the adult animal.

EPAS1, a recently cloned bHLH/PAS-domain transcription factor highly related to HIF1α, was shown to strongly activate the construct HHNS when both plasmids were cotransfected into HEK293 cells (17). We have reproduced this result, and from our preliminary studies, it seems that intronic sequences outside the 1.7-kb enhancer are necessary for this strong induction. This provides further evidence for the existence of additional intrinsic transcriptional regulatory sequences.

Furthermore, we have presented evidence that the structure of the 1.7-kb enhancer is complex. An internal 303-bp fragment was identified as sufficient to confer EC-specific activity to the heterologous tk promoter. However, this construct (tkNcXb) was even more integration-site-dependent and more responsive to ectopic activation than the 1.7-kb enhancer-driven construct (tkXX), suggesting that elements necessary for reproducible expression of transgenes, such as a matrix attachment region (18), existed outside the 303-bp region but within the 1.7-kb enhancer.

Several putative transcription factor binding sites, whose presence and functionality have previously been demonstrated in other genes, were identified in the core enhancer sequence: YY1 sites have been implicated in activation (19) and repression (20) of transcription. αINF2 sites have been suggested to play a role in the induction of interferon-β gene expression by viruses (21). GATA sites are known target sites for transcriptional regulators of genes such as von Willebrand factor (22) and endothelial nitric oxide synthase (23), and GATA-2 has been shown to be expressed of genes involved in tissue remodeling (31). Furthermore, Ets1 is expressed in migrating and sprouting ECs during embryonic and tumor angiogenesis (31). Close to this Ets1 site, a hexameric palindrome (GAGCTC) has been identified. Interestingly,
in the tumor necrosis factor α promoter, the same sequence also lies close to an Ets1 site, where it is part of a putative binding site for the bZIP transcription factor complexes AP-1 or CREB/ATF (32, 33), which cooperate with the Ets1 site in the induction of this promoter by phorbol esters (33). It can therefore be speculated that a bZIP-related transcription factor complex activates the TIE2 intronic enhancer via this hexamer, perhaps in synergy with Ets1.

Head-to-tail clustered PEA3 sites occur in the promoter of TIE1 and TIE2, in regions highly conserved among species (ref. 7 and data not shown). These conservations of the clustered PEA3 sites in both TIE1 and TIE2 gene may imply their importance for the EC-specific gene transcription, although single PEA3 sites, such as that present in the intronic TIE2 enhancer, have also been demonstrated to be functional (34). Besides PEA3, which belongs to the Ets family of transcription factors, Ets1 itself was previously shown to be a candidate for PEA3-site binding (35).

Our mutational analysis of the intronic enhancer showed that the sites for INF2α, GATA, and Sp1 are not critical for in vivo activity at day E11.5. On the other hand, the phenotype of the mutation affecting the NF-S site and the octameric palindrome homologous to the TIE1 promoter suggests that this region contributes to the integration-site independency of the 1.7-kb enhancer. However, the endothelial expression was only slightly weakened by this mutation.
Deletion of the 33-bp SacI-XbaI fragment of the core enhancer, which included the sites for bZIP, CP2γ, and PEA3, and mutation of the Ets1 site just 5′ of the SacI site abolished in vivo activity of the enhancer completely. Preliminary Ets1-cotransfection experiments in endothelial (BAECs) and non-endothelial cells (HEK293) caused only a slight induction of reporter gene expression (tXXK). However, this would be expected if either Ets1 was not a limiting factor or these cell systems lacked putative cofactors.

Identification and characterization of the transcription factors involved in the EC-specific expression of the TIE2 gene should lead to a better understanding of the transcriptional mechanisms of the EC-specific expression of TIE2 and other genes expressed in ECs. This type of molecular analysis should also contribute to the understanding of the mechanisms of EC lineage establishment and differentiation and the development of therapies to perturb certain unwanted gene expression in endothelial cells.

Furthermore, by substitution of the LacZ reporter gene in the described expression vectors with other genes such as dominant negative or dominant active mutants, the function of virtually any gene in ECs of transgenic mice can be challenged in vivo. In addition, genes that are usually only expressed under certain conditions such as during an inflammatory response can now be constitutively expressed, and the resulting phenotypes can be analyzed in vivo.

Establishment of an inducible system (36, 37), based on the regulatory sequences described in this report, enabling the switching on and off of transgenes specifically in ECs, could allow investigation into the role of genes with an early lethal knock-out phenotype, such as the vascular endothelial growth factor receptors (38, 39) or TIE2 itself (40, 41) during later stages of development and in adult animals.

Futhermore, the transgenic mouse lines described herein will be a valuable tool for several applications. For instance, the specific sorting of LacZ-positive cells from tissues, which seems feasible from our preliminary experiments, could help to address the differential gene expression of ECs in vivo, which underlies their enormous heterogeneity and plasticity. Also, the endothelial LacZ activity may be a useful marker for the grade of vascularization of a solid tumor of a cancer mouse model, a parameter difficult to assess by other means but important to evaluate the efficacy of antiangiogenesis therapies (for review, see ref. 42). We believe that the transgenic expression vector and transgenic mouse lines described in this report should prove useful in both basic and clinical studies of the cardiovascular system.

Note Added in Proof. The TIE2/LacZ Tg mouse (182.30) reported here is now deposited in the Induced Mutant Resource of the Jackson Laboratory (Bar Harbor, ME) for requests.

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References