Identification of an octamer element required for *in vivo* expression of the *TIE1* gene in endothelial cells

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TIE1, an endothelial-cell-specific tyrosine kinase receptor, is required for the survival and growth of microvascular endothelial cells during the capillary sprouting phase of vascular development. To investigate the molecular mechanisms that regulate the expression of *TIE1* in the endothelium, we analysed transgenic mouse embryos carrying wild-type or mutant TIE1 promoter/LacZ constructs. Our data indicate that an upstream DNA octamer element (5′-ATGCAAAT-3′) is required for the *in vivo* expression of *TIE1* in embryonic endothelial cells. Transgenic embryos carrying the wild-type TIE1 promoter (−466 to +78 bp) fused to LacZ and spanning the octamer element demonstrate endothelial-cell-specific expression of the reporter transgene. Point mutations introduced within the octamer element result in a significant decrease of endothelial LacZ expression, suggesting that the octamer site functions as a positive regulator for *TIE1* gene expression in endothelial cells. DNA–protein binding studies show that the octamer element exhibits an endothelial-cell-specific pattern of binding via interaction with endothelial-cell-restricted factor(s). Our findings suggest an important role for the octamer element in regulating the expression of the TIE1 receptor in the embryonic endothelium and suggest a common mechanism for the regulation of the angiogenic and cell-specific *TIE1* and *TIE2* genes during vascular development.

Key words: angiogenesis, transcription, vascular development.

INTRODUCTION

Development of the vascular system in vertebrates is a complex process involving the differentiation, recruitment and assembly of several cell types into structurally and functionally normal blood vessels [1,2]. Endothelial cells play an essential role in this developmental process. In this role, they express a variety of cell-specific and non-specific surface receptors that interact with ligands secreted by nearby mesenchymal cells. Two such receptors, TIE1 and TIE2, initiate and co-ordinate developmental events in the latter stages of vascular development. TIE1, an orphan tyrosine kinase receptor, is required for maintaining the survival and growth of microvascular endothelial cells during capillary sprouting [3–5]. TIE2, a receptor for the angiopoietin family of ligands, regulates several processes, including sprouting angiogenesis, remodelling of the vasculature into larger and smaller vessels and recruitment of peri-endothelial cells to the vessel wall [3,6,7].

Recent progress in understanding the transcriptional basis of vascular development has allowed the identification and characterization of a number of DNA elements involved in regulating the expression of vascular developmental genes in endothelial cells [8–10]. Using the murine TIE2 promoter as a model, we have shown previously that the *in vivo* expression of *TIE2* in the endothelium requires the presence of an intact octamer element in its 5′-flanking region [10]. Similar *in vivo* studies using a TIE1 promoter construct have shown that a minimal 900 bp regulatory region is sufficient to confer specific reporter transgene expression in embryonic endothelial cells [11]. This region contains several putative protein-binding sites, including Ets sites, as well as a consensus octamer element. Mutations within the Ets site and the octamer element resulted in a significant loss of TIE1 promoter activity in cultured cells [12]. *In vivo* studies using a promoter construct lacking one of the 5′ Ets sites revealed a reduction, but not an abolition of the embryos showing significant β-galactosidase activity in endothelial cells [12]. These data suggest that, whereas the Ets motif is likely to regulate TIE1 promoter activity, additional elements are likely to possess such activity within the promoter construct. Importantly, the role of the octamer element in regulating *TIE1 in vivo* remains to be examined. This is particularly important since previous studies indicate that the octamer element functions as an enhancer of the TIE2 promoter in endothelial cells. A number of indicators suggest that *TIE2* and *TIE1* may share common mechanisms for cell-specific gene expression in the endothelium. Both genes have the same onset of expression at 8.5 days of embryonic development (E8.5) [13], both have a pattern of expression that is restricted to endothelial cells [13] and both share a common vascular developmental function [3–5]. This has prompted us to determine whether the TIE1 promoter also requires the octamer element for normal transcriptional activity in endothelial cells.

Using wild-type and mutant TIE1/LacZ transgenic constructs, we show in the present study that the octamer element is indeed required for cell-specific activity of the TIE1 promoter. Whereas the wild-type construct allows targeting of LacZ expression in embryonic endothelial cells, the construct containing mutations in the octamer element fails to do so. Furthermore, DNA–protein binding studies indicate that the TIE1 octamer element exhibits an endothelial-cell-specific pattern of binding and that the cell-specific band consists of a ternary complex between the octamer element, the transcription factor Oct1 (octamer-binding protein 1) and an endothelial cell cofactor. Taken together, our findings

Abbreviations used: EMSA, electrophoretic mobility-shift assay; Py-4-1 cells, polyoma-transformed murine endothelial cells; bEND.3 cells, murine brain microvascular endothelial cells; RAW cells, murine macrophage cells; Namalwa cells, human Burkitt lymphoma B-cells; CCL-1 cells, murine fibroblast cells; MEL cells, murine erythroleukaemia cells; YSC cells, murine yolk sac cells; X-Gal, 5-bromo-4-chloroindol-3-yl β-D-galactopyranoside; Oct1, octamer-binding protein 1.

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indicate that the octamer motif serves as a cell-specific positive regulatory element for the TIE1 promoter and suggest a central role for this element in mediating the expression of angiogenic receptors in the embryonic endothelium.

EXPERIMENTAL

Reporter gene constructs

Two DNA constructs were used to generate transgenic mice, TIE1.wt.LacZ and TIE1.mut.LacZ. TIE1.wt.LacZ consists of 544 bp of murine *TIE1* regulatory sequences, comprising 466 bp of 5′-flanking sequence and 78 bp of 5′-untranslated region, subcloned upstream of the *lacZ* gene. TIE1.mut.LacZ is identical to TIE1.wt.LacZ except for point mutations introduced in the octamer site (ATGCAAAT → ATGCAAGC), known not to allow interaction with octamer-binding proteins [10]. Site-directed mutagenesis of the octamer element was made using the Altered Sites II *in vitro* mutagenesis kit according to the manufacturer's instructions (Promega).

For functional studies of the octamer element in association with the TIE1 promoter in cultured endothelial cells, the 544 bp (−466 to +78) wild-type and mutant constructs were subcloned upstream of the luciferase gene in a pGL2 Basic vector (Promega) to yield p(−466)tie1.luc and p(−466)mtie1.luc respectively. For the TIE2 promoter, a DNA fragment consisting of 753 bp of 5′-flanking sequence and 318 bp of 5′-untranslated region and containing the octamer element was subcloned upstream of the luciferase gene in a pGL2 Basic vector to yield p(−753)tie2.luc. An identical DNA construct, except for mutations in the octamer element that were similar to those introduced into the *TIE1* octamer sequence, yielded p(−753)mtie2.luc.

Cell culture and DNA-transfection studies

Polyoma-transformed murine endothelial cells (Py-4-1), murine brain microvascular endothelial cells (bEND.3), murine macrophage cells (RAW), human Burkitt lymphoma B-cells (Namalwa), murine fibroblast cells (CCL-1) and murine erythroleukaemia cells (MEL) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Murine yolk sac cells (YSC) were grown in α-minimal essential medium supplemented with 18% fetal bovine serum. Transfection of Py-4-1 cells was performed using the calcium phosphate method as described in [14]. DNA mixture was composed of 1 μg of vector and 1 μg of simian virus 40/LacZ vector as an internal control. Cell lysates were assayed for luciferase and β-galactosidase activities [15]. Transfections were performed in triplicate and conducted a minimum of three times.

Production of transgenic mice, genotyping embryos and β-galactosidase staining

The TIE1.wt.LacZ and TIE1.mut.LacZ constructs were purified by agarose-gel electrophoresis followed by Geneclean (Bio 101). Each DNA was then suspended in injection buffer (10 mM Tris/HCl, pH 7.4/0.1 mM EDTA) and passed through a 0.45 μl filter (Millipore). The morning following the appearance of the vaginal plug was designated day 0.5 post-coitus. Microinjection into oocytes and surgical procedures were performed as described previously [16]. Pregnant mice were killed at E10.5 and the embryo, yolk sac and decidua were isolated.

To genotype embryos, PCR amplification of 340 bp of the *lacZ* gene was performed on yolk-sac tissue using the primers 5′-TACCACAGCGGATGGTTCGG-3′ and 5′-GTGGTGGTTA-TGCCGATCGC-3′ under the following conditions: (i) denaturation at 94 °C for 1.5 min, (ii) denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min and extension at 72 °C for 1 min for 30 cycles and (iii) final extension at 72 °C for 10 min. PCR buffer consisted of 50 mM KCl, 10 mM Tris/HCl, pH 8.3, 1.5 mM $MgCl_2$ and 0.01% (v/v) gelatin. Amplification products were separated on a 2% (w/v) agarose gel and visualized with ethidium bromide [10].

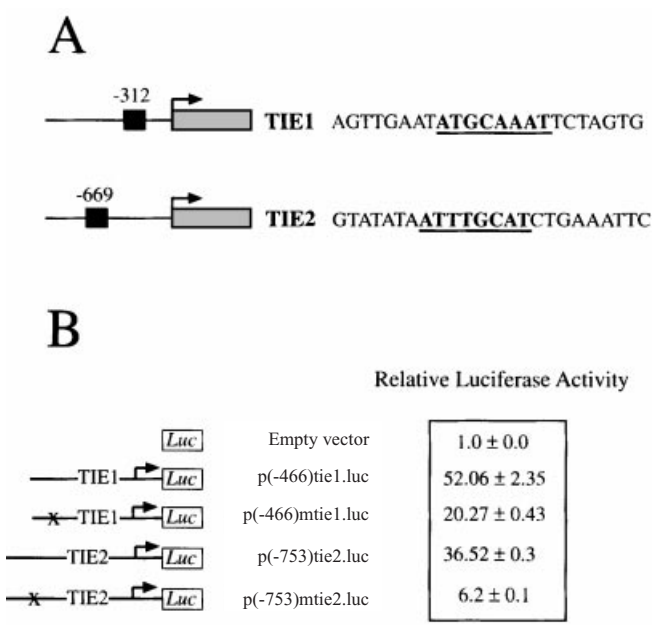

Figure 1 ***In vitro*** **analysis of wild-type and mutant TIE1 promoter activity in endothelial cells**

(**A**) Diagram of the murine TIE1 and TIE2 promoters. The consensus octamer element, 5′-ATGCAAAT-3′, and its complimentary sequence are located in the 5′-flanking regions of *TIE1* and *TIE2* respectively, at −312 bp and −669 bp respectively. The octamer elements are represented by black boxes. The first exon of each gene is represented by a box above which is an arrow, indicating the transcription start site. The nucleotide sequence of the oligonucleotides used in DNA-binding studies are given, with the octamer motif highlighted in bold and underlined. (**B**) Diagram of the relative transcriptional activities of the wild-type and mutant *TIE1* and *TIE2* constructs in cultured endothelial cells. Plasmids p(−466)tie1.luc and p(−753)tie2.luc contain wild-type promoter fragments −466 bp of *TIE1* and −753 bp of *TIE2* respectively. Plasmids p(−466)mtie1.luc and p(−753)mtie2.luc contain the same fragments mutated at the octamer element. Data are means ± S.D. of luciferase activity in light units normalized to β-galactosidase activity from three different experiments performed in triplicate.

For whole-mount LacZ staining, embryos were washed twice in PBS, fixed for 30 min in 0.2% (v/v) glutaraldehyde and rinsed three times in a solution containing 0.1 M phosphate buffer, pH 7.3, 2 mM $MgCl_2$, 0.01% (w/v) sodium deoxycholate and 0.02% (v/v) Nonidet P-40 [16]. Embryos were stained in a rinse solution containing 1 mg/ml 5-bromo-4-chloroindol-3-yl β-D-galactopyranoside (X-Gal; Roche), 5 mM $K_3Fe(CN)_6$ and 5 mM $K_4Fe(CN)_6$ for a period of 4 h. Embryos were then mounted in paraffin, sectioned, deparaffinized, rehydrated, counter-stained with Nuclear Fast Red (Zymed), dehydrated, mounted in Permount (Fisher) and photographed under dark-field or bright-field microscopy.

Electrophoretic mobility-shift assay (EMSA)

Nuclear extracts from the various cell types were prepared according to the method by Schreiber et al. [17]. Oligonucleotides spanning the octamer-binding sites of the *TIE1* and *TIE2* genes were used in the EMSA (Figure 1A). Oligonucleotides were end-

labelled with [γ-^{32}P]ATP (NEN) and binding conditions consisted of 40 mM NaCl, 27 mM KCl, 10 mM Hepes, pH 7.9, 0.5 mM EDTA, 0.75 mM dithiothreitol, 4% (v/v) Ficoll, 2% (v/v) glycerol, 2 μg of poly(dI-dC) and 20 ng/μl herring sperm DNA [10]. Nuclear extracts (6 μg) were incubated at room temperature for 10 min in the binding buffer. Labelled oligonucleotides (100–200 pg) and, when indicated, a 100-fold excess of unlabelled oligonucleotides were added and reactions incubated at room temperature for an additional 20 min. For supershift assays, 3 μg of rabbit polyclonal anti-Oct1 antibodies (Santa Cruz Biotechnology) or rabbit IgG were pre-incubated with nuclear extracts for 30 min at room temperature prior to the addition of labelled oligonucleotides. Samples were then loaded on to a 5% (w/v) polyacrylamide gel and electrophoresed in 1 × Tris/glycine buffer at 200 V for 5 h at 4 °C.

RESULTS

The octamer element is required for the transcriptional activity of the TIE1 promoter in cultured endothelial cells

Analysis of the 5′-flanking region of the murine *TIE1* gene reveals the presence of a consensus octamer element, 5′-ATGCAAAT-3′, located at −312 bp from the transcription start site (Figure 1A) [11,12]. Its DNA sequence is complementary to that of the *TIE2* octamer element required for the *in vivo* expression of *TIE2* in endothelial cells [10].

To examine the role of the octamer element in mediating the transcriptional activity of the TIE1 promoter, we conducted a series of reporter-gene transfection studies in cultured endothelial cells. Two DNA constructs, p(−466)tie1.luc and p(−753)tie2.luc, were designed to contain the respective lengths of the TIE1 and TIE2 promoters, extending 5′ of the octamer element of each gene and encompassing the transcription start sites. Point mutations in each octamer element resulted in p(−466)mtie1.luc and p(−753)mtie2.luc, respectively. The wild-type p(−753)tie2.luc construct was used as a positive control because earlier *in vivo* and *in vitro* studies indicate that it is transcriptionally active in endothelial cells [10,15].

Transient transfection of murine Py-4-1 cells with the p(−466)tie1.luc construct resulted in a more than 50-fold increase in relative luciferase activity in comparison with the promoterless vector (Figure 1B). The *TIE2* construct p(−753)tie2.luc was similarly active, resulting in a 37-fold increase in relative luciferase activity. Transfection of the mutant octamer construct p(−466)mtie1.luc resulted in a 66% reduction of the reporter gene activity compared with the *TIE1* wild-type construct. As expected, transfection of the p(−753)mtie2.luc also resulted in a significant loss of TIE2 promoter activity in endothelial cells. These findings indicate that a *TIE1* DNA construct spanning 544 bp of regulatory sequences is transcriptionally active in cultured endothelial cells and that, similar to *TIE2*, an intact octamer element is required for full promoter activity.

The octamer element is required for *in vivo* transcriptional activity of the TIE1 promoter in embryonic endothelial cells

To examine the role of the octamer motif in mediating the *in vivo* expression of the *TIE1* gene, we conducted a series of experiments in transgenic mice. These studies were aimed at evaluating the endothelial expression of a *lacZ* reporter gene under the control of a wild-type or mutant *TIE1* construct. For this purpose, we designed a wild-type construct, TIE1.wt.LacZ, that consists of the 544 bp *TIE1* fragment (−466 to +78) subcloned upstream of the *lacZ* gene flanked by a nuclear localization signal and a poly-A tail. The mutant construct TIE1.mut.LacZ is identical to the wild-type construct except for point mutations introduced in the octamer element that interfere with proteins that bind the octamer. The *TIE1* regulatory region used to generate these constructs differs slightly from the one used by Korhonen et al. [11]. Whereas both sequences have a common 5′ end at an *Afl*II site located at −466 bp, our construct has a shorter 3′ end extending only to +78 bp. Since the TIE1 promoter mediates reporter transgene expression mainly in embryonic endothelial cells [11,12] and since native *TIE1* expression is first detected at E8.5 [13], we analysed LacZ expression in embryos at E10.5.

Table 1 Summary of the activity of the wild-type and mutant TIE1/LacZ constructs in transgenic embryos

Embryos carrying the wild-type and mutant transgenic constructs were generated, genotyped and stained to examine LacZ expression at E10.5 as described in the Experimental section. For the mutant TIE1/LacZ construct, a smaller percentage of transgenic embryos (29%) showed evidence of LacZ expression than the wild-type embryos (60%).

	Total number of embryos	Number of transgenic embryos	Embryos showing LacZ staining
Wild-type construct	60	20 (33% of total)	12 (60% of transgenic)
Mutant construct	68	31 (46% of total)	9 (29% of transgenic)

We analysed 60 first-generation embryos carrying the wild-type TIE1.wt.LacZ construct, and 33% of the embryos incorporated the transgene, as demonstrated by PCR of yolk sac tissue using *lacZ*-specific primers. Of the transgenic embryos, 60% demonstrated LacZ staining that was restricted to endothelial cells (Table 1). 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 stained positively (Figure 2a). The intensity and extent of LacZ expression varied among the transgenic embryos, with some exhibiting stronger staining than others. Even in the embryos that showed the most extensive LacZ staining, expression in endothelial cells was not uniform since most, but not all, endothelial cells were stained. These findings correlate with previous data by Korhonen et al. [11]. None of the transgenic embryos showed evidence of ectopic non-endothelial staining. Taken together, these data indicate that the 544 bp regulatory region of *TIE1* contains DNA-regulatory element(s) that are sufficient for cell-specific expression of the TIE1 promoter in embryonic endothelial cells. However, in a similar manner to the TIE2 promoter construct [10], additional elements outside the upstream promoter region appear to be required for a full and uniform expression in the endothelium.

We collected 68 transgenic embryos carrying the mutant construct TIE1.mut.LacZ and analysed them in a manner identical to the wild-type embryos. Of these embryos, 46% incorporated the transgene, as demonstrated by PCR of the embryonic yolk sac. However only 29% of the transgenic embryos demonstrated LacZ staining as compared with 60% for the wild-type embryos (Table 1). Furthermore, the pattern and intensity of LacZ expression in the embryos carrying the mutant construct differed significantly from those carrying the wild-type construct. In the embryos that showed evidence of β-galactosidase expression, the staining was mostly ectopic and non-vascular (Figure 2b, panels A, B, C and F). This is probably the result of the loss of endothelial-cell enhancer activity, allowing the *lacZ* gene to fall under the control of a nearby genomic enhancer. In the remaining embryos that exhibited vascular

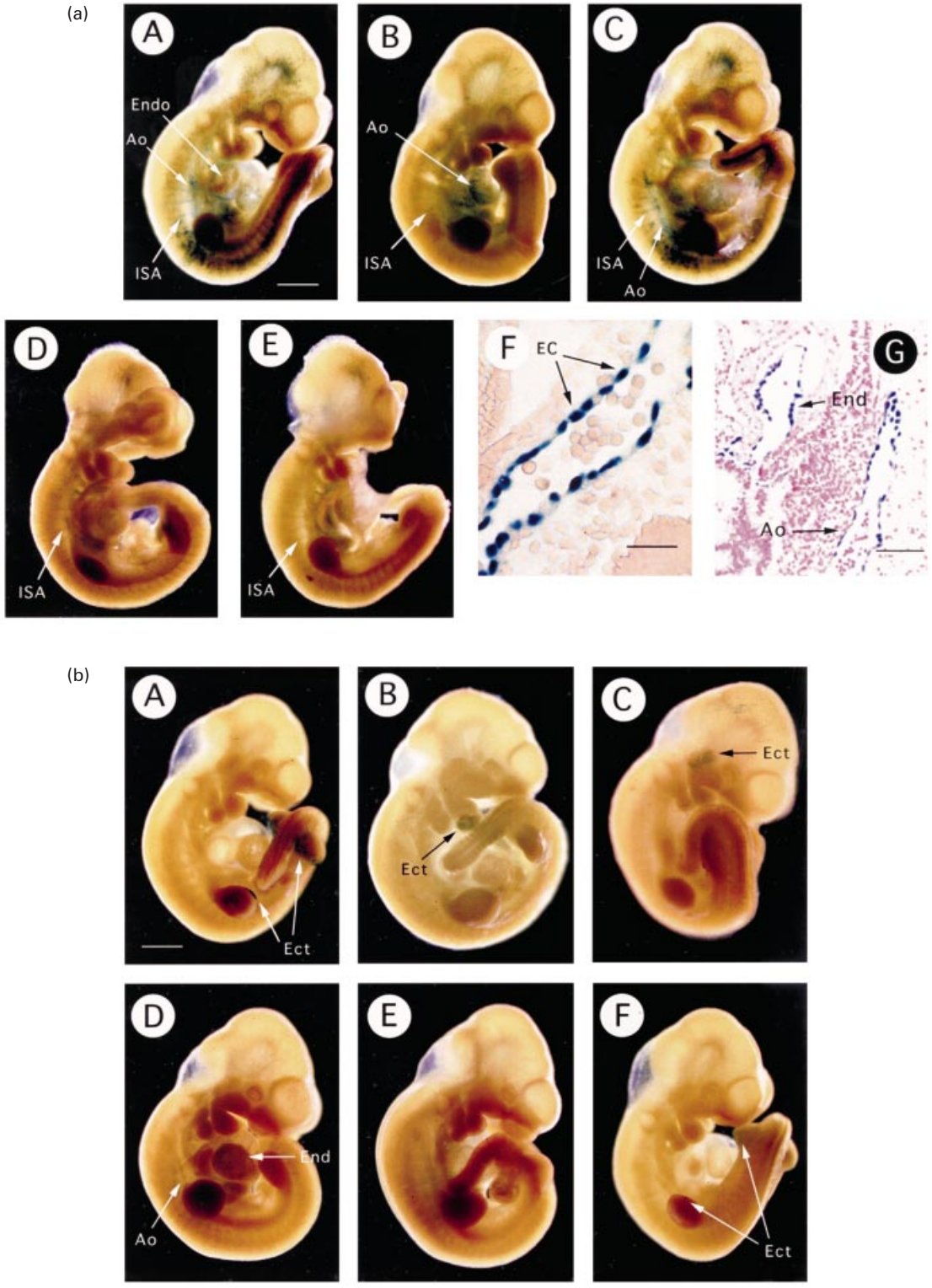

Figure 2 ***In vivo*** **analysis of wild-type and mutant** ***TIE1*** **promoter activity in endothelial cells**

(**a**) Reporter-gene expression mediated by the wild-type *TIE1* promoter in endothelial cells. A DNA construct (TIE1.wt.LacZ) containing the *lacZ* reporter gene under the control of wild-type *TIE1* regulatory sequences was used to generate transgenic E10.5 embryos. These embryos were stained with X-Gal to evaluate β-galactosidase expression. (A–E) Whole-mount staining of five embryos showing LacZ expression in the dorsal aorta (Ao), intersomitic arteries (ISA) and endocardium (Endo). The level and extent of LacZ expression varied among the embryos; however, expression was restricted to the embryonic vasculature and endocardium (scale bar, 500 μm). (F and G) Cellular localization of LacZ staining. Parasagital sections of the dorsal aorta (F; scale bar, 30 μm) and middle section of the embryo (G; scale bar, 60 μm) allow the identification of LacZ-stained endocardial (End) and endothelial (EC) cells. There is no evidence of ectopic non-endothelial staining. (**b**) Reporter-gene expression mediated by the mutant *TIE1* promoter in endothelial cells. Embryos carrying the mutant construct TIE1.mut.LacZ are stained for β-galactosidase expression. (A–F) Whole-mount staining shows low-level and patchy LacZ expression in the vascular bed in comparison with embryos carrying the wild-type construct. Embryos show different patterns of transgene activity and exhibit either ectopic LacZ expression (A–C and F) or low-level and patchy expression in the intersomitic arteries and endocardium (D, E; scale bar, 500 μm). Ect, ectopic.

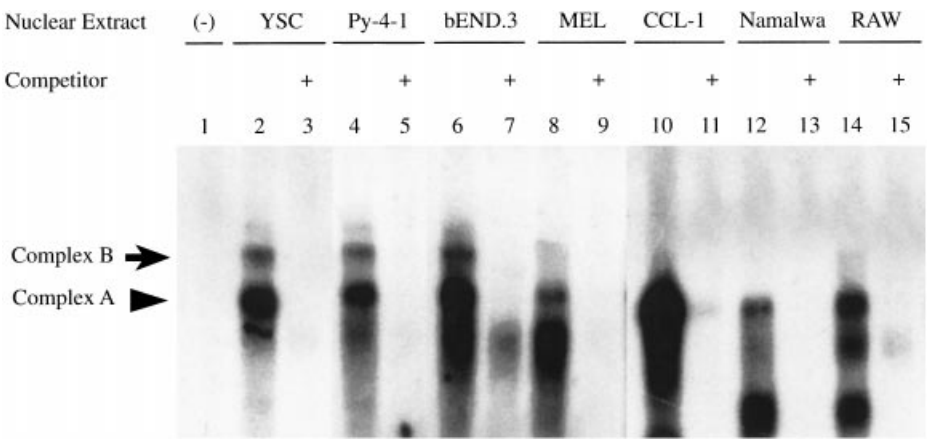

Figure 3 EMSA using an oligonucleotide probe encoding the *TIE1* octamer element

Endothelial cell types included YSC cells (lanes 2 and 3), Py-4-1 cells (lanes 4 and 5) and bEND.3 cells (lanes 6 and 7). Non-endothelial cell types included MEL cells (lanes 8 and 9), CCL-1 cells (lanes 10 and 11), Namalwa cells (lanes 12 and 13) and RAW cells (lanes 14 and 15). Lane 1 contains no nuclear extracts. A retarded band, complex A, was noted in all cell types. A slower-migrating band, complex B, was only noted in endothelial cells, indicating an endothelial-cell-specific pattern of binding.

staining, patchy and weak staining was limited to some endothelial cells within the aorta, intersomitic arteries and endocardium. Of nine mutant construct embryos that stained positively for LacZ, only two demonstrated weak staining in the aorta and endocardium (Figure 2b, panels D and E), and the number of stained endothelial cells and their level of expression was significantly less than that observed with the wild-type construct embryos. Taken together, these data indicate that a mutation of the octamer element markedly impairs reporter-gene expression and ablates most of the transcriptional activity of the TIE1 promoter in endothelial cells. These findings support the hypothesis that the octamer element is required for normal expression of *TIE1* in the embryonic endothelium.

The octamer elements of *TIE1* and *TIE2* display identical and cell-specific patterns of binding

To determine whether the *TIE1* octamer element binds endothelial-cell-restricted factor(s), an EMSA was performed using nuclear extracts from a number of endothelial and non-endothelial cell types. The endothelial cell lines included YSC, Py-4-1 and bEND.3 cells, whereas the non-endothelial cell lines included MEL, CCL-1, Namalwa and RAW cells. All three endothelial cell types demonstrated two retarded bands, complexes A and B (Figure 3). All four non-endothelial cell types demonstrated only one of the retarded bands, complex A (Figure 3). These findings are similar to those noted previously with the *TIE2* octamer element [10], suggesting that complex B, the slower of the two bands, results from the specific interaction between the octamer element and protein factor(s) present exclusively in endothelial cell extracts. These findings correlate with *in vivo* data and suggest a mechanism for endothelial-cell-specific expression of *TIE1* mediated by the octamer element.

Characterization of the endothelial-cell-specific band

Using the *TIE2* octamer element in an EMSA, we have shown previously that complex A represents the binding of the ubiquitous transcription factor Oct1, whereas complex B represents the binding of a protein complex consisting of Oct1 and an endothelial-cell-specific cofactor [10]. To determine whether a similar mechanism is at work with the *TIE1* octamer element, experiments were conducted to characterize the nature of these two complexes. EMSA was performed using the *TIE1* octamer element and nuclear extracts derived from the endothelial cell type Py-4-1 and the fibroblast cell line CCL-1 in the presence of an anti-Oct1 antibody (Figure 4). Since complex A has a ubiquitous pattern, it was thought to represent the binding of the transcription factor Oct1 to the octamer element. The addition of anti-Oct1 antibodies resulted in the supershift of this complex in both Py-4-1 and CCL-1 cells (Figure 4, lanes 3 and 7), whereas addition of control IgG had no effect (Figure 4, lanes 4 and 8).

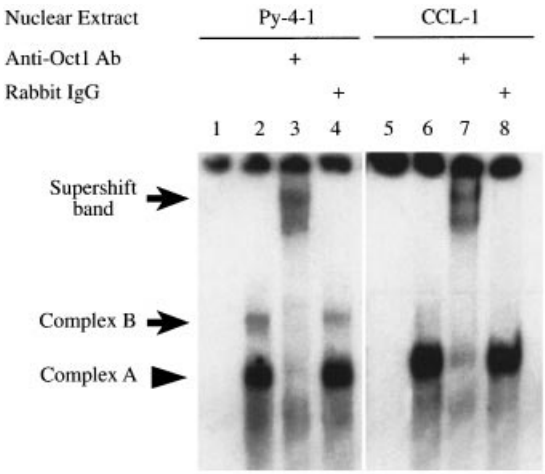

Figure 4 Antibody supershift assay using the *TIE1* octamer oligonucleotide as a probe

Nuclear extracts (6 μg) were derived from murine Py-4-1 endothelial cells and the murine fibroblast cell line CCL-1. Lanes 1 and 5 represent control reactions without nuclear extracts. Lanes 2 and 6 represent reactions without antibodies. The addition of anti-Oct1 antibodies resulted in a supershift of complex A (lanes 3 and 7), indicating that it represents the binding of the ubiquitously expressed factor Oct1 to the octamer element. The endothelial-cell-specific complex B was also supershifted (lane 3) indicating that it contains Oct1. No supershift was noted with the addition of rabbit IgG (lanes 4 and 8).

The addition of anti-Oct1 antibodies also resulted in supershift of the endothelial-cell-specific complex B (Figure 4, lane 3), indicating that this complex also contains Oct1. These findings are identical to those observed with the *TIE2* octamer element [10], indicating that complex B results from the interaction of Oct1 and an endothelial cell cofactor with the octamer element. This suggests a role for an Oct1 cofactor in mediating the expression of the *TIE1* and *TIE2* genes in embryonic endothelial cells.

DISCUSSION

The identification of endothelial-cell receptors and their ligands has greatly improved our understanding of the contribution of cell-surface molecules to the process of vascular development in the vertebrate embryo. In contrast, little is known of the intracellular pathways that are involved in vascular development. Recent studies from several laboratories are providing important data of the transcriptional mechanisms that regulate the expression of the TIE2 receptor, TIE1 receptor and vascular endothelial growth factor receptor 2 ('VEGF-R2') in endothelial cells [8–12,18]. Among these developmental genes, *TIE2* has been the most extensively studied with regard to its transcriptional regulation. Initial *in vitro* studies of the TIE2 promoter have allowed the identification of a number of positive regulatory elements [15]. However, transgenic experiments showed that these elements lie outside the regulatory region that confers endothelial-cell-specific expression [8,9]. More recent data from our laboratory [10] indicate that an octamer element located in the 5′-flanking region is required for the *in vivo* expression of *TIE2* in embryonic endothelial cells. DNA–protein binding studies showed that this element binds a protein complex consisting of the ubiquitous transcription factor Oct1 and an endothelial-cell-restricted cofactor [10]. Interaction of this protein complex with the octamer element appears to confer tissue specificity to the expression of *TIE2* in the embryonic endothelium.

TIE1, another member of the TIE family of receptors, has also been the subject of studies aimed at characterizing the transcriptional mechanisms that mediate its expression. In a series of experiments, Korhonen et al. [11] and Iljin et al. [12] were able to successfully target reporter-gene expression under the control of upstream *TIE1* regulatory sequences to the endothelial cells of the mouse embryo. *In vivo* transgenic studies indicated a possible role for an Ets-binding site in mediating the cell-restricted expression. However, the role of an octamer element located just 3′ to the Ets site, which was found to possess *in vitro* transcriptional activity, was not examined *in vivo*. Since previous studies from our laboratory indicated an important role for such elements in the *in vivo* expression of *TIE2*, we aimed to further characterize its transcriptional activity within the TIE1 promoter. Since both TIE1 and TIE2 are first expressed simultaneously in endothelial cells of the mouse embryo and possess a common developmental function, a possibility exists that a common mechanism mediates their expression.

To examine the role of the octamer motif in the expression of *TIE1*, we studied the minimal regulatory region that spans this element and which is known to confer endothelium-specific gene expression in transgenic embryos [11,12]. Here we show that the expression of a reporter transgene under the control of this regulatory region requires the presence of an intact octamer element. Mutation of this element, designed to prevent protein binding, resulted in a quantitative and qualitative impairment of reporter-gene expression *in vivo*. Specifically, there was a significant decrease in the level and extent of endothelial LacZ staining, fewer embryos showed positive staining (29 % for the mutant compared with 60 % for the wild-type construct) and ectopic non-endothelial expression was evident in embryos carrying the mutant *TIE1* construct. In a similar manner to our observations with the TIE2 promoter, we show that the octamer element is also required for optimal transcriptional activity of the TIE1 promoter in endothelial cells. Despite significant similarities, some differences exist between reporter-transgene expression from the TIE1 and TIE2 promoters. Whereas both promoter constructs could confer an endothelial-cell-specific pattern to the reporter transgene [8,10–12], LacZ expression was slightly less extensive and involved a smaller number of endothelial cells in the case of the *TIE1* construct. These findings suggest that additional DNA elements located outside the regulatory region under study are required for full activity of the TIE1 promoter in endothelial cells. Since *TIE1* and *TIE2* have a simultaneous onset of expression at E8.5 and the transgenic mice were analysed at E10.5 and E9.5, respectively, our data suggest that the octamer element contributes to the positive regulation of both genes at this early stage of embryonic development [10]. Since the Ets-binding site appears to have a role in the expression of *TIE1*, it remains to be seen whether the octamer and Ets elements collaborate to mediate the cell-specific enhancer activity to the *TIE1* promoter in the developing endothelium.

Even though the expression of *TIE1* and *TIE2* is restricted to the endothelium, these factors have an overlapping, but not identical, spatial expression in the various vascular beds, reflecting endothelial-cell heterogeneity. They also display a distinct temporal pattern of expression since *TIE2*, but not *TIE1*, is down-regulated in the endothelium following birth [13]. Whereas our observations indicate a requirement for the octamer element at an early developmental stage, further studies are needed to determine whether it is required for the expression of *TIE1* and *TIE2* at later developmental stages and into adulthood. Additional DNA elements are likely to account for the spatial and temporal differences in expression between *TIE1* and *TIE2*. This highlights the complexity of the transcriptional mechanisms that regulate the expression of vascular developmental genes in endothelial cells.

In summary, our data suggest that the transcriptional activity of the TIE1 and TIE2 promoters requires the presence of an intact octamer element, and that this element interacts with a protein complex consisting of the ubiquitous transcription factor Oct1 and an endothelial-cell-restricted cofactor. A similar scenario of cell- and gene-specific expression involving the octamer element, Oct1 and a cell-restricted cofactor is the activation of immunoglobulin genes in B-cells. In this case, an octamer element located in the promoter region of all immunoglobulin genes mediates antigen-induced expression of immunoglobulin through the interaction with a protein complex consisting of Oct1 and the cell-restricted cofactor Bob1/OCA-B/OBF-1 [19–22]. In view of the likely existence of a common precursor for the endothelial and haematopoietic lineages, the haemangioblast, it is possible that endothelial cells and B-cells have maintained a common mechanism of cell-specific expression mediated by the octamer motif, Oct1 and a cell-restricted Oct1 co-activator. Identification of the endothelial-cell Oct1-cofactor should significantly improve our understanding of the transcriptional mechanisms that regulate the early stages of vascular development in vertebrates.

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