Targeting the Hprt locus in mice reveals differential regulation of Tie2 gene expression in the endothelium

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To study the differential regulation of the murine Tie2 gene expression in the endothelium, we have targeted the hypoxanthine phosphoribosyltransferase (Hprt) gene to generate two single-copy transgenic mice: T1, containing the 2,100-bp Tie2 promoter upstream from the β-galactosidase (LacZ) gene, and T5, which also included an enhancing element originating from the first intron of the Tie2 gene. Comparing T1 and T5 embryos at day E10.5 revealed differential endothelial cell-specific expression of LacZ, whereas colocalization analyses showed that the expression was confined to endothelial cells. Moderate reporter gene activity was observed in the brain and kidney of T1 adults, whereas extensive LacZ gene expression was seen in the vasculature of most organs of the T5 adults. This study demonstrates the feasibility of targeting the Hprt locus with endothelial cell-specific sequences to analyze the spatial-temporal expression of transgenes. Of particular importance is the observation that the analysis of a single transgene copy in a defined locus allows for an accurate and rapid comparison of transcriptional activity among regulatory DNA sequences.

Keywords: endothelial cells; development; transgenesis; homologous recombination; gene regulation

The endothelial cell-specific tyrosine kinase receptor Tie2 (also known as Tek) is involved in the remodeling of blood vessels, the interactions of endothelial cells with extracellular matrix and perivascular cells (3, 13), and the sprouting and growth of vessels from preexisting vasculature (angiogenesis) (13). In addition to its role in early vascular development, this receptor is expressed in adulthood, suggesting a role in vascular maintenance (17). The knowledge that Tie2 is upregulated under pathological processes such as tumor growth and wound healing has evoked a large interest in the biology of this receptor (11, 12, 17). Abolishing Tie2 function by knocking out the receptor (3, 13) or its recently identified activating ligand (2, 16) results in embryonic lethality in mice between embryonic days E9.5 and E10.5 (3, 13, 16). This knowledge indicates the importance of understanding the mechanisms of Tie2 gene regulation during (patho)physiological processes.

To date, our knowledge of the regulation of the murine Tie2 gene in vivo has depended on transgenesis by injection of DNA into the pronucleus of oocytes. Briefly, these analyses have shown that regulatory sequences, which comprise 2.1 kb upstream of the start codon of the Tie2 gene, can promote endothelial cell-specific expression of LacZ in embryonic development (14, 15). In addition, sequences located in intron 1 of the Tie2 gene were reported to be required for endothelial-specific transgenic expression throughout adulthood (14). Furthermore, it was shown that an octameric element in the Tie2 promoter is required for expression of LacZ in early embryonic development (4). These studies have largely contributed to the knowledge of transcriptional regulation of the Tie2 gene; however, by using conventional methods of generating transgenics, these investigators had to account for the random integration of one or multiple copies of their transgene into the genome, site integration dependency, and the occurrence of ectopic staining. These problems were more prevalent for the weaker promoter/enhancer elements and required analysis of multiple mouse lines to draw conclusions about their transcriptional activity (14).

To study the in vivo regulation of the Tie2 gene in a controlled fashion, we have explored a targeting system developed by Bronson et al. (1). By employing an embryonic stem (ES) cell line lacking a functional X-linked Hprt gene, these investigators were able to analyze a single copy of a transgene at a defined locus. The lack of this gene product causes the cells to die when grown on selective media containing hypoxanthine, aminopterin, and thymidine (HAT). However, reconstitution of this locus, using a specific targeting vector, allows for a direct selection of a homologous recombination event, such that only properly targeted ES cells survive the HAT selection. The presence of a multiple doing site (MCS) in this vector allows for easy insertion of transgenic sequences between the 5′ homology sequences and the Hprt sequences (1). To test this method, Bronson et al. (1) integrated the murine bcl-2 cDNA in the Hprt locus under the control of the human and chicken β-actin promoter enabling direct comparative analyses of their respective expression patterns. When integrated upstream of a functional Hprt gene, the β-actin promoters directed expression of...
the transgene in a wide variety of tissues. Moreover, the expression of regulatory sequences targeted to the Hprt locus closely resembled endogenous activity.

Together with the accompanying study performed by Guillot et al. (6), we demonstrate that the Hprt targeting system is a powerful tool for the in vivo analysis of endothelial cell-specific promoters. This technology may prove valuable both in the search for and the functional analysis of DNA sequences required for endothelial cell-specific expression. Also, it will be valuable in unraveling the complexity of gene regulation during (patho)physiological processes such as tumor-induced angiogenesis, psoriasis, tissue remodeling following injury, and diabetic retinopathy. Finally, the speed with which Hprt-targeted animals can be generated in combination with overall low production costs, compared with pronuclear injections of DNA into oocytes, may promote its further employment.

MATERIALS AND METHODS

Targeting

Constructs. The two plasmids used to construct the Hprt targeting vectors were kindly provided by Urban Deutsch and Thorsten Schlaeger (Max Planck Institute, Bad Nauheim, Germany) (14). The first plasmid comprised a 2.1-kb murine Tie2 promoter fragment (flanked by two Hind III restriction sites), followed by the LacZ reporter gene, and a simian virus 40 (SV40) poly(A) signal sequence. The second plasmid included a 1.7-kb portion of intron 1 of Tie2 directly downstream of the poly(A) sequence.

The vector that was used to target the Hprt locus (pMP8SKB) was a generous gift from Sarah Bronson (Pennsylvania State University, College of Medicine). To facilitate the construction of our final vectors, we first modified the MCS of pMP8SKB such that, in addition to Not I and Mlu I, it also included unique sites for Sfi I and Pme I (pMP8I). The following MCS was introduced into pUC19: 5′-EcoRI, Pme I, Sac I, Kpn I, Xma I, Bam HI, Xba I, Sal I, Pst I, Sfi I, Not I, Hind III 3′ (pUC19-3). After isolating the Tie2-LacZ cassette from the plasmids, these DNA fragments were subcloned into pUC19-3 followed by directional cloning into pMP8I. The final constructs, denoted pMP8I-T1 (2.1-kb Tie2 promoter, LacZ) and pMP8I-T5 (2.1-kb Tie2 promoter, LacZ, 1.7-kb Tie2 enhancer), were verified by restriction analysis and sequencing. The restriction enzyme Sac I was used to linearize the targeting constructs prior to electroporation into ES cells.

Cell lines and culture The BK4-ES cell line (derived from a 129/Ola strain mouse; Ref. 9) was provided by Sarah Bronson. This ES cell line, with a male karyotype, contains a disrupted locus for the Hprt gene on the X chromosome, characterized by the lack of the promoter and exons 1 and 2 of the Hprt gene. Culturing was performed with standard ES cell procedures. For the electroporation of one construct, two 10-cm dishes of BK4 cells were trypsinized, washed twice with HBS (25 mM HEPES, pH 7.1, 134 mM NaCl, 5 mM KCl, and 0.7 mM Na2HPO4) and counted. Special care was taken to prevent any cell differentiation, including the growth of only small colonies, frequent pipetting to ensure single cell suspension when splitting the cells, and changing the media 2 h prior to any manipulation. Seven to fifteen micrograms of purified, linearized DNA and 5–7 × 10⁴ ES cells in 0.8 ml HBS were mixed by inversion inside the electroporation cuvettes (Gene Pulser Cuvettes, 0.4 cm electrode gap, 10; Bio-Rad Laboratories, Hercules, CA) and incubated on ice for 10 min. The cells were then electroporated at 230 V and 500 μF, then at 240 V and 500 μF (Gene Pulser Apparatus; Bio-Rad), incubated on ice for 10 min, and plated on a single 10-cm petri dish with 2.5 × 10⁵ gamma-irradiated murine embryonic fibroblasts (feeders) seeded the day before. Media containing 0.1 mM hypoxanthine, 0.0004 mM aminopterin, and 0.016 mM thymidine (LY-HAT; Sigma, St. Louis, MO) was introduced after allowing the cells to recover for 14–16 h. The media was changed daily during the selection of homologous recombinants. Shiny, regularly shaped, individual colonies, observed 9–10 days after electroporation, were picked under a dissection hood into an empty 96-well plate. Clones were exposed to 0.25% trypsin/1 mM EDTA (GIBCO BRL; Life Technologies, Rockville, MD) for 5 min at 37°C. The cells were resuspended and transferred to a 96-well plate, presseed with 3 × 10⁴ feeders/cm². After 6–8 days of propagation and having been split into 24-well plates, individual colonies were frozen in 10% DMSO and 90% FBS and used for the isolation of DNA (10). Using PCR, we screened the colonies for the targeted transgenic cassette, and positive clones were used for injection into blastocysts.

Mice

Generation of transgenic mice. The generation of chimeras was achieved by microinjection of properly targeted ES cells into C57BL/6 (B6; Taconic, Germantown, NY) blastocysts as described previously (8). Male chimeras with an estimated 80–90% brown coat color were bred to B6 females to obtain agouti offspring (agouti pups are 129/B6 FL hybrids that have received the 129 ES cell genome). Female agouti offspring were bred to B6 to obtain homozygous female mice.

Genotyping. The offspring of the agouti female/B6 male cross were genotyped using PCR. Ear punches (of 2- to 6-wk old mice) were incubated overnight at 55°C in a total volume of 100 μl proteinase K buffer (50 mM KCl, 10 mM Tris·HCl, pH 8.3, 2.0 mM MgCl2, 0.1 mg/ml gelatin, 0.45% Nonidet P-40) containing 1 mg/ml proteinase K (Sigma). One microliter of this mix was used as template in a multiplex PCR reaction (two sets of primers). One set of primers, generating a product of ~400 base pairs (bp), was used to monitor the presence of the targeted allele. The upstream primer, complementary to the Hprt promoter region (of human origin), was part of the targeting vector (5′-CCT GAT CCT TAA GCC CCC AC-3′). The downstream primer was complementary to the 5′ end of the Tie2 promoter (5′-CCC TGC TGT CAT CCC CC-3′). The second set of oligonucleotides was designed to screen for the murine wild-type Hprt allele and generated a product of 191 bp (5′-GGG ATT ACA CAT ATG TGT CGC CAC-3′ and 5′-CTA CCC AGA CAA TTA AGA TCT TTC-3′). PCR was performed in a 10-μl volume using the Clontech Advantage2 PCR enzyme system kit (Clontech Laboratories, Palo Alto, CA) on a Robocycler (Stratagene, La Jolla, CA) with the following conditions: 2 min at 94°C followed by thirty cycles of 30 s at 94°C, 1 min at 63°C, 30 s at 72°C, ending with a 2-min extension at 72°C.

Embryo analysis. Given the unpredictability of natural matings, embryos were obtained through surgical transfers from targeted donor mice (crossed to targeted males) to pseudopregnant female mice to maximize the number of embryos for each of the analyses (8). Embryos were harvested on day E10.5, and the yolk sacs were removed for genotyping (see above). After washing in cold PBS, the embryos were fixed for 10 min in ice-cold, freshly prepared 4% formaldehyde/PBS. After thoroughly rinsing in cold PBS, the embryos were stained in PBS containing 0.02% Nonidet P-40, 0.01% SDS, 2 mM MgCl₂, 5 mM K-ferricyanide, 5 mM K-ferrocyanide, and 0.016 mM thymidine (LY-HAT; Sigma, St. Louis, MO) was introduced after allowing the cells to recover for 14–16 h. The media was changed daily during the selection of homologous recombinants. Shiny, regularly shaped, individual colonies, observed 9–10 days after electroporation, were picked under a dissection hood into an empty 96-well plate. Clones were exposed to 0.25% trypsin/1 mM EDTA (GIBCO BRL; Life Technologies, Rockville, MD) for 5 min at 37°C. The cells were resuspended and transferred to a 96-well plate, presseed with 3 × 10⁴ feeders/cm². After 6–8 days of propagation and having been split into 24-well plates, individual colonies were frozen in 10% DMSO and 90% FBS and used for the isolation of DNA (10). Using PCR, we screened the colonies for the targeted transgenic cassette, and positive clones were used for injection into blastocysts.
1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; Boehringer Mannheim, Indianapolis, IN). X-Gal staining was performed for 18 h at 30°C in 6-cm petri dishes on a shaking platform (low speed). After staining, the embryos were rinsed in PBS and photographed using Kodachrome 100 ASA film.

Colocalization studies in paraffin sections. For colocalization purposes, X-Gal-stained embryos were dehydrated, embedded in paraffin, and sectioned (10 µm). Deparaffinization was performed using xylene baths, 100% ETOH, and 95% ETOH. After the slides were washed for 5 min in running water, endogenous hydrogen peroxidase was inactivated by incubation in 0.5% hydrogen peroxide (Sigma) in water. Slides were rinsed with water and blocked for 2 h at 4°C with blocking buffer (PBS containing 1% BSA and 0.05% saponin). Primary antibodies were incubated overnight at 4°C. Rat anti-mouse PECAM-1 polyclonal antibody (PharMingen, San Diego, CA) was used at a 1:100 dilution. Rat anti-human thrombomodulin (TM; Ref. 5) was used at a final concentration of 30 ng/ml. Rabbit anti-human von Willebrand factor (vWF) (Dako) was used at a working concentration of 1:100. Control sections were incubated without a primary antibody. After overnight incubation, the slides were washed in PBS followed by the application of biotinylated anti-rat IgG (PECAM-1 and TM) or biotinylated anti-rabbit IgG (vWF) in blocking buffer (see above). The buffer was supplemented with 5% rat serum (PECAM-1 and TM) or 5% rabbit serum (vWF) and secondary antibodies (Vectastain Elite ABC kits; Vector Laboratories, Burlingame, CA). After a 1-h incubation at room temperature, the slides were washed in PBS and incubated in 0.5% hydrogen peroxide/PBS for 10 min at room temperature and processed according to the recommendations of the manufacturer. Staining was performed in the absence of NiCl₂ to render a brown precipitate and was typically aborted after 3- to 10-min incubation at room temperature. The slides were photographed directly.

Analysis of adult tissues. Two- to six-week-old mice were killed using intraperitoneal injections of an overdose of 2.5% Avertin (prepared from a 100% stock solution of 10 g 2,2,2-tribromoethyl alcohol/10 ml tert-amyl alcohol; Sigma). Heart, lung, liver, kidney, spleen, and brain were harvested and rinsed twice in cold PBS and then fixed for 2 h in freshly prepared 4% formaldehyde/PBS (on ice). After the fixation, the organs were rinsed in PBS and placed in 30% sucrose/PBS overnight at 4°C. With use of a bath of dry ice and isopentane, tissues were embedded in Cryomatrix medium (Shandon, Pittsburgh, PA) and stored at −80°C. Sections of 4–10 µm (ProbeOn Plus slides; Fisher Scientific, Fair Lawn, NJ) were kept on dry ice and subsequently stored at −80°C.

In performing the X-Gal staining of sectioned tissues, slides were removed from the freezer and directly submerged in 3% formalin (Sigma) in PBS for 30 min at 4°C. Slides were rinsed in PBS and incubated overnight in a freshly prepared X-Gal solution (see above) and counterstained with Eosin Y (Sigma). After dehydration, the slides were mounted with cover slips using Cytoseal XYL (Stephen Scientific, Riverdale, NJ) and allowed to set for 2 h prior to visual analysis and photography.

RESULTS

Generation of Hprt-Targeted Animals

It took 17 days to obtain correctly targeted BK4 cell clones that were ready for injection into blastocysts (from the day of electroporation; see Fig. 1). Specifically, we expanded four clones for construct pMP8II-T1 and

![Fig. 1. Hprt targeting system. Female agouti mice with a single copy of a transgene in the Hprt locus can be generated in 4.5–5 mo (from the day of the electroporation). This time scale is based on our experience that most of the embryonic stem (ES) cell clones will contribute to the germ line in the first litter (when breeding chimeric males to C57BL/6 mice). HAT, media containing hypoxanthine, aminopterin, and thymidine.](http://physiolgenomics.physiology.org)
five clones for pMP8II-T5, which after DNA analysis all proved to contain the targeted transgenic cassettes (results not shown). Sixty percent of chimeric males (with a 90–95% agouti coat color) crossed with B6 females produced agouti offspring in either their first or second litter (i.e., the ES cells contributed to the germ line). This was true for both the T1 and T5 plasmids, as well as a multitude of other Hprt targeting constructs (unpublished data). From the pMP8II-T1 and the pMP8II-T5 plasmids, we generated one mouse line of each denoted T1 and T5, respectively. Overall, the Hprt targeting system made it possible to analyze 4-wk-old heterozygous female animals within 4.5–5.0 mo (see Fig. 1).

Embryo Analysis

Using female agouti mice and their male chimeric parent, a total number of 26 T1 and 27 T5 embryos were obtained. Analyzed independently, staining was observed in more than 50% of the embryos. These results were in agreement with our PCR-based genotyping. Consistently, for each line, the embryos with one targeted allele in absence of a wild-type allele (male hemizygotes) showed a stronger overall staining than embryos with both a targeted Hprt allele and a wild-type allele (female heterozygotes). The latter observation reflected the process of lyonization in females, i.e., the random inactivation of one of the X chromosomes in cells of various tissues (1). The staining pattern among embryos from the same mouse line was consistent.

The embryos depicted in Fig. 2 are representative of the T1 and T5 lines. Whole mount X-Gal staining of embryos (E10.5) of both lines revealed uniform staining of endothelial beds in absence of ectopic reporter gene activity. However, we identified clear differences in expression patterns when comparing the two lines (see Fig. 2): T1 embryos showed β-galactosidase expression in the dorsal aorta, the umbilical veins, the intersomital vessels (but only close to the point where they branched off the dorsal aorta), the aortic arches, the heart, the liver, the visceral pouch in between the first and second aortic arch, and the major vessels in the brain. Overall, the Tie2 promoter-driven expression of LacZ (in the absence of the intronic 1.7-kb Tie2 enhancer) at this stage of development appeared to be confined to the major vessels (see Fig. 2). More extensive LacZ expression was observed in the T5 embryo compared with the T1 animals. This is illustrated by extensive staining of sprouting vessels in the brain (Fig. 2D) and an increased reporter gene activity in the intersomital vessels which extended ventrally (Fig. 2B). Also, LacZ expression was observed in the caudal veins, vessels that did not stain in the T1 embryos (Fig. 2B).

Colocalization Studies

To verify that the X-Gal precipitate in the embryos was indeed confined to endothelial cells, we performed colocalization studies of X-Gal with endothelial cell-specific markers, i.e., PECAM-1, TM, and vWF. These analyses were carried out using paraffin sections of whole mount X-Gal-stained T1 embryos. The results showed clear colocalization of X-Gal precipitate with the brown reaction product of the hydrogen peroxidase activity linked to the secondary antibodies (see Fig. 3). The antibodies directed against the cell surface markers (i.e., PECAM-1 and TM) gave a brown precipitate enclosing the blue cytoplasmic staining of the LacZ-expressing cells. In contrast, the vWF antibody reaction stained the already blue-stained cytoplasm of positive endothelial cells resulting in an almost black coloration of the cytoplasm.

Fig. 2. Representative X-Gal-stained embryos at embryonic day 10.5 (E10.5). A: a T1 embryo (2.1-kb Tie2 promoter, LacZ). B: a T5 embryo (2.1-kb Hprt allele and a wild-type allele (female heterozygotes). The latter observation reflected the process of lyonization in females, i.e., the random inactivation of one of the X chromosomes in cells of various tissues (1). The staining pattern among embryos from the same mouse line was consistent. The embryos depicted in Fig. 2 are representative of the T1 and T5 lines. Whole mount X-Gal staining of embryos (E10.5) of both lines revealed uniform staining of endothelial beds in absence of ectopic reporter gene activity. However, we identified clear differences in expression patterns when comparing the two lines (see Fig. 2): T1 embryos showed β-galactosidase expression in the dorsal aorta, the umbilical veins, the intersomital vessels (but only close to the point where they branched off the dorsal aorta), the aortic arches, the heart, the liver, the visceral pouch in between the first and second aortic arch, and the major vessels in the brain. Overall, the Tie2 promoter-driven expression of LacZ (in the absence of the intronic 1.7-kb Tie2 enhancer) at this stage of development appeared to be confined to the major vessels (see Fig. 2). More extensive LacZ expression was observed in the T5 embryo compared with the T1 animals. This is illustrated by extensive staining of sprouting vessels in the brain (Fig. 2D) and an increased reporter gene activity in the intersomital vessels which extended ventrally (Fig. 2B). Also, LacZ expression was observed in the caudal veins, vessels that did not stain in the T1 embryos (Fig. 2B).
Analysis of Adult Tissues

Tissues from organs of various ages (2, 5, and 6 wk old) did not demonstrate substantial staining differences when each of the lines was analyzed. Independent X-Gal staining of male and female tissues of both lines generated reproducible results (not shown), although we observed less transgene expression in the female heterozygotes compared with hemizygous males as the result of lyonization. For a direct comparative analysis, we chose to kill 4-wk-old hemizygous males of both lines, after which the organs, tissues, and sections were processed in parallel. The low-magnification photographs in Fig. 4 are representative of the expression patterns observed in the various tissues. High-power photographs of the brain (T1), lung (T5), and the spleen (T5) (see Fig. 5) are provided to further illustrate the endothelial-cell specificity of transgene expression.

Tissues of 4-wk-old T1 hemizygous males (expressing one copy of the transgene in every cell) exhibited X-Gal precipitate in glomeruli of the kidney (Fig. 2A) and the larger vessels of the brain (Fig. 2C). In contrast, expression was absent in the heart (Fig. 2E), the lung (Fig. 2G), the spleen (Fig. 2I), and the liver (not shown), but on rare occasions we observed very weak expression in some larger vessels of the heart, the spleen, and the kidney (not shown).

The T5 counterpart presented with far more extensive β-galactosidase activity (see Fig. 4), particularly in the kidney (Fig. 4B), brain (Fig. 4D), heart (Fig. 4F), lung (Fig. 4H), and spleen (Fig. 4J), whereas, again, the liver was essentially free of any X-Gal precipitate (data not shown). The T5 animals showed strong expression in the kidney vasculature, including larger and smaller veins and arteries, and a more intense staining of the endothelial cells in the glomeruli (Fig. 4B) compared with T1. The expression pattern of the transgene in the brain of the T5 animals included the staining of more and smaller vessels compared with T1 (Fig. 4D). The T5 heart presented with strong transgene activity in coronary arteries, veins, and some smaller blood vessels (Fig. 4F). We did not observe any X-Gal staining in the lung of the T1 mice (Fig. 4G), but the addition of 1.7-kb enhancer fragment to the transgenic cassette in the T5 animals drove widespread expression of the transgene in the capillaries and larger vessels of the lung (Fig. 4H). Finally, the spleen in the T5 line exhibited strong reporter gene expression in both smaller and larger vessels (Fig. 4J).

DISCUSSION

To understand the transcriptional regulation of the murine Tie2 gene during (pathophysiological) angiogen-
esis, we have successfully employed a previously described targeting strategy (1). This methodology involves targeting of the Hprt gene locus in ES cells, the subsequent generation of chimeric animals, and germ line transmission of the targeted transgenes. It has allowed for a direct comparison of the in vivo activity of two different Tie2 promoter/enhancer LacZ cassettes by the integration of a single copy in the same genomic locus. The two constructs differed only by the presence or absence of a downstream enhancer element originating from intron 1 of the Tie2 gene. Our data demonstrate that the Tie2 promoter alone drives endothelial-specific expression of LacZ in both embryonic development, and in the brain and kidney of the adult. Moreover, the enhancer appears to contain regulatory elements that are required for gene expression during angiogenesis in

Fig. 4. Adult tissues of the T1 (left, Tie2 promoter-LacZ) and T5 (right, Tie2 promoter-LacZ-enhancer) lines. Processing and sectioning of the organs of 4-wk-old male hemizygotes of both lines were performed in parallel. Left: kidney (A), brain (C), heart (E), lung (G), and spleen (I) of the T1 adult. Right (B, D, F, H, and J): same organs of the T5 adult. Arrows in C and D indicate positively stained vessels, to emphasize the heterogeneity of LacZ expression in brain of T1 and T5 lines. Photographs were taken at the following optical magnification settings: kidney, \( \times 200 \); brain, \( \times 100 \); heart, \( \times 100 \); lung, \( \times 200 \); and spleen, \( \times 100 \). High-power inset (\( \times 1,000 \)) in A shows LacZ-positive cells in glomeruli of T1 adult animal. The inset in A represents a different glomerulus from that indicated on right in A.
Spatial-Temporal Expression of Tie2

Our reexamination of the in vivo activity of Tie2 regulatory sequences agrees in part with the data obtained by others through conventional transgenesis (14, 15). For example, in the T1 animals, we confirmed that the 2.1-kb Tie2 promoter is endothelial cell-specific in embryonic development. Furthermore, in both cases, transgene expression in E10.5 T1 embryos was largely confined to endothelial beds arising from de novo formation of blood vessels (vasculogenesis) (7, 14). However, our analysis of adult T1 tissues revealed LacZ expression in endothelial cells in the brain and in the glomeruli of the kidney. This contrasts with the data from Schlaeger et al. (14), who reported that additional regulatory sequences were required for expression through adulthood while they specifically mentioned the absence of staining in the glomeruli. Also, these investigators did not show the extensive staining of sprouting vessels during angiogenesis in transgenic embryos containing the intronic enhancing element which our T5 embryos displayed. The absence of ectopic staining is another important difference in the comparative analysis of their conventional transgenic animals with Hprt-targeted mice. These discrepancies result from the use of different transgenic systems, because the same DNA sequences were used. We assume that the observed effects in this study result from the incorporated Tie2 sequences. This is supported by reports of retained endogenous specificity of regulatory sequences targeted to the Hprt locus (1, 6). Furthermore, the differences in observed expression patterns between the T1 and T5 lines could not have been the result of site integration dependency or the number of integrated copies of the transgene. Nevertheless, we cannot rule out the occurrence of interactions between regulatory elements of the Tie2 gene and sequences in the Hprt promoter.

Compared with the T1 line, T5 displayed more extensive reporter gene activity in E10.5 embryos. This differential expression was most evident in the caudal veins and in endothelial cells of small vessels generated by angiogenic processes. This is best illustrated by the staining of sprouting vessels in the developing brain. In addition, we observed that the staining of the intersomital vessels extended further toward the dorsal part of the somites compared with the T1 embryos. However, the data did not indicate that the expression of LacZ in the vasculature was enhanced, i.e., more intensely stained, compared with the T1 animals. Still, our results support the idea that the addition of the enhancer regulatory sequences, i.e., the 1.7-kb fragment of intron 1 of the Tie2 gene, expanded the expression of the transgene to additional blood vessels during development. T1 and T5 both demonstrated a lack of endothelial cell-specific staining in the adult liver. It is of interest to note that Bronson et al. (1) also reported absence of transgene expression in the liver, an organ where the Hprt gene itself is expressed. These findings suggest that the expression of the transgenic cassette is largely unaffected by the activation of the intact Hprt promoter. However, these observations also indicate that the Hprt strategy might not be suited, in general, for studying regulation of gene expression in the liver. The expressions of LacZ in both the lung and the spleen of the T5 animals were in sharp contrast to the lack of expression in T1 adults. However, our data do not allow us to draw conclusions as to whether this is due to a general enhancing effect of the 1.7-kb DNA fragment or the presence of organ-specific regulatory elements within this intronic sequence. Also, the current analysis of each of the lines clearly shows intravascular heterogeneity of Tie2 regulation as illustrated by the presence of X-Gal precipitate in only a portion of the vasculature in each organ. The staining pattern of vascular beds was reproducible, demonstrating that the heterogeneous expression of the marker gene was not due to stochastic enzymatic activity β-galactosidase. The significance of intravascular differences in...
the transcriptional regulation of the Tie2 gene, particularly with regard to gene activation during pathological conditions, is at present unclear and requires further investigation.

**Hprt Targeting, Retained Tissue Specificity, and Efficacy**

We have taken advantage of the Hprt targeting system to study endothelial cell-specific gene regulation. Our colocalization analyses, using various endothelial cell-specific markers in two different Hprt-targeted lines, have shown that the Tie2 promoter retains its natural endothelial cell specificity in both early embryonic development and adulthood. Also, the vascular staining patterns observed in the embryos and the adult tissues were reproducibly uniform and consistent. These results agree with the predictions of Bronson et al. (1), who hypothesized that specific promoters and enhancers could be expected to retain their natural specificity, because the Hprt gene is ubiquitously expressed in virtually all developmental stages in all tissues, which indicates its presence in a constitutively active chromatin region.

In addition to the retained tissue specificity, this method proved to be a fast and cost-efficient approach. Figure 1 illustrates that Hprt-targeted transgenic mice can be generated in a short period of time because of a consistent contribution of the BK4 ES cells to the germ line. This observation is based on our extensive experience in generating Hprt-targeted lines. By scaling down the ES cell work to only one tissue culture dish per construct and expanding only a few clones, we have reduced the costs involved in establishing these lines. However, the real reduction in cost lies in the generation and maintenance of only a small number of mouse lines per construct compared with multiple lines of conventional transgenic animals. Bronson et al. (1) have shown essentially invariant expression levels of their transgene in multiple ES cell lines and subsequent mouse lines, illustrating the sufficiency of only one line per construct. Also, Guillot et al. (6) have shown that multiple Hprt-targeted mouse lines obtained with the same targeting construct presented with the same expression pattern. In addition, these investigators report that the orientation of the transgenic cassette does not alter expression patterns of their transgene.

**Conclusions**

Using the Hprt targeting system, we have shown that the 2.1-kb murine Tie2 promoter drives endothelial cell-specific expression in vessels, not in only early embryonic development but also in the brain and the kidney of the adult mouse. In addition, our data indicate that the previously described 1.7-kb enhancing element, derived from intron 1 of the Tie2 gene, is activated during angiogenesis in E10.5 embryos and also drives extensive transgene expression in the adult vasculature (14).

This report, in combination with the accompanying report of Guillot et al. (6), has shown that targeting the Hprt locus provides an efficient alternative methodology to investigate tissue-specific promoters in endothelial cells. Also, it could be valuable to study the effects of tissue-specific expression of various gene products during physiological processes. The ability to compare animals carrying single-copy transgenes in the same locus allows for a very accurate in vivo comparison of regulatory sequences. This, in conjunction with the speed and efficiency of this system, may accelerate searches for tissue-specific regulatory elements as well as facilitate the dissection of spatial-temporal expression patterns of target genes.

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