Targeting of human eNOS promoter to the Hprt locus of mice leads to tissue-restricted transgene expression

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Guillot, Pascale V., Lixin Liu, Jan Albert Kuivenhoven, Jason Guan, Robert D. Rosenberg, and William C. Aird. Targeting of human eNOS promoter to the Hprt locus of mice leads to tissue-restricted transgene expression. Physiol Genomics 2: 77–83, 2000.—Phenotypic heterogeneity of the endothelium arises from cell type-specific differences in gene expression. An understanding of the mechanisms that underlie differential gene expression would provide important insight into the molecular basis of vascular diversity. In standard transgenic assays, multiple copies of heterologous DNA cassettes are randomly integrated into the mouse genome, resulting in significant line-to-line variation in expression. To overcome these limitations, we have targeted a single copy of a transgene that contains 1,600 bp of the human endothelial nitric oxide synthase (eNOS) promoter coupled to the LacZ reporter gene to the X-linked hypoxanthine phosphoribosyltransferase (Hprt) locus of mice by homologous recombination. The transgene was inserted in either of the orientations relative to that of the Hprt gene. In mice derived from multiple embryonic stem (ES) cell clones, the expression pattern was limited to a subset of endothelial cells, cardiomyocytes, and vascular smooth muscle cells. These findings suggest that Hprt locus targeting is a feasible tool for studying endothelial cell-restricted gene regulation.

One way to overcome the limitations inherent in standard transgenesis is to insert a single copy of the transgene into a predetermined site of the genome by homologous recombination. In a recent study, promoter-reporter gene constructs were successfully targeted to the X-linked Hprt locus. This locus has two important advantages. First, a functional Hprt gene is lacking in the parent embryonic stem (ES) cell and can be reconstituted by homologous recombination. As a result, targeted ES cells are selectable in medium containing hypoxanthine, aminopterin, and thymidine (HAT). Second, since the Hprt gene is ubiquitously expressed, the locus is presumably relaxed and transcriptionally favorable. In other words, transgenes that are inserted into the locus are likely to be free of the constraints of higher order gene regulation. In the above report, an Hprt-targeted transgenic construct containing the β-actin promoter coupled to the β-globin-targeted LacZ transgene to the Hprt locus in both orientations. Analysis of mice derived from independent ES cell clones has revealed colocalization of the LacZ target gene and the endogenous eNOS gene in a subset of endothelial cells, cardiomyocytes, and vascular smooth muscle cells. These results show that Hprt locus targeting is a feasible tool for studying endothelial cell-restricted gene regulation in the context of the cell’s native microenvironment. Until recently, this goal was most readily achieved by the introduction of promoter-reporter gene constructs into the germ line of mice by standard transgenic techniques. However, this approach is limited by the unpredictable effect of copy number and integration site on expression levels and patterns. Without the means to control for these variables, multiple independent lines of mice must be analyzed to reach statistically significant conclusions. In the final analysis, the high costs of generating and maintaining transgenic animals may preclude detailed promoter analysis in vivo.

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ENDOTHELIAL CELL PHENOTYPES reflect the unique needs of the underlying tissue (7, 11, 19). Despite a wealth of knowledge about how endothelial cells differ from one another, remarkably little is known about the molecular basis of vascular diversity. One approach to this problem is to study the mechanisms that underlie endothelial cell subtype-specific transcriptional regulation. In previous studies, we have shown that the expression of endothelial cell-specific genes such as von Willebrand factor and endothelial nitric oxide synthase (eNOS) is regulated by distinct mechanisms in different subsets of endothelial cells (1, 2, 8, 13). Moreover, we have determined that vascular bed-specific expression of these gene products is ultimately controlled by signals that reside in the extracellular milieu (2, 13). These observations underscore the importance of studying endothelial cell gene regulation in the context of the cell’s native microenvironment. Until recently, this goal was most readily achieved by the introduction of promoter-reporter gene constructs into the germ line of mice by homologous recombination. However, this approach is limited by the unpredictable effect of copy number and integration site on expression levels and patterns. Without the means to control for these variables, multiple independent lines of mice must be analyzed to reach statistically significant conclusions. In the final analysis, the high costs of generating and maintaining transgenic animals may preclude detailed promoter analysis in vivo.

One way to overcome the limitations inherent in standard transgenesis is to insert a single copy of the transgene into a predetermined site of the genome by homologous recombination. In a recent study, promoter-reporter gene constructs were successfully targeted to the X-linked Hprt locus. This locus has two important advantages. First, a functional Hprt gene is lacking in the parent embryonic stem (ES) cell and can be reconstituted by homologous recombination. As a result, targeted ES cells are selectable in medium containing hypoxanthine, aminopterin, and thymidine (HAT). Second, since the Hprt gene is ubiquitously expressed, the locus is presumably relaxed and transcriptionally favorable. In other words, transgenes that are inserted into the locus are likely to be free of the constraints of higher order gene regulation. In the above report, an Hprt-targeted transgenic construct containing the β-actin promoter coupled to the β-globin-targeted LacZ transgene to the Hprt locus in both orientations. Analysis of mice derived from independent ES cell clones has revealed colocalization of the LacZ target gene and the endogenous eNOS gene in a subset of endothelial cells, cardiomyocytes, and vascular smooth muscle cells. These results show that Hprt locus targeting is a feasible tool for studying endothelial cell-restricted gene regulation.
reliable assay system with which to study cell-type-specific gene regulation.

MATERIALS AND METHODS

Targeting vectors and eNOS constructs. A DNA fragment containing a 1,600-bp human eNOS 5’ flanking sequence coupled to the coding region of LacZ was inserted into the Hprt targeting vector, pMP85SKB (a generous gift from Sarah Bronson, Pennsylvania State University, College of Medicine) in the same transcriptional orientation as the Hprt gene (forward eNOSlaclacZ-Hprt) as well as in the opposite orientation (reverse eNOSlaclacZ-Hprt). To generate the forward construct, the 1600eNOS lacZ plasmid (13) was digested with Kpn I, blunt-ended with Klenow polymerase and dNTPs, and then digested with Not I to release the Bluescript vector (Stratagene, La Jolla, CA). The resulting DNA fragment was ligated into the Hprt targeting vector digested with Mlu I (blunt-ended) and Not I. To generate the reverse construct, the 1600eNOSI lacZ plasmid was digested with Not I, blunt-ended with Klenow polymerase and dNTPs, and then digested with Kpn I. The promoter-LacZ fragment was ligated into the EcoRI V site of the Bluescript vector. The resulting plasmid, eNOSI lacZ/pBlue, was digested with Xho I, blunt-ended with Klenow polymerase and dNTPs, and then digested with Not I. The resulting promoter-LacZ fragment was ligated into the Hprt targeting vector digested with Mlu I (blunt-ended) and Not I. The eNOSI lacZ/Hprt targeting constructs were linearized with Sal I prior to electroporation.

Cell culture and electroporation. The BK4-ES cells were a generous gift from Sarah Bronson (6). ES cells were grown on gamma-irradiated murine embryonic fibroblasts in high-glucose DMEM (Life Technologies, Gaithersburg, MD) supplemented with 15% heat-inactivated FBS, 1% L-glutamine, 1% glucose DMEM (Life Technologies, Gaithersburg, MD). HAT-resistant colonies were picked 10 days later for propagation.

Generation of transgenic mice. Targeted ES cells were injected into C57BL/6-derived blastocysts which were then transferred into the uteri of recipient Swiss Webster females (see Ref. 9 for details). Resulting chimeric males were bred with C57 females, and the F1 agouti female offspring was mated with wild-type C57BL/6 females. Vaginal plugs were checked each morning. The day of appearance of a vaginal plug was designated at day 0.5 postcoitus (E0.5). The females were killed, and embryos were removed from uteri for analysis at day E10.5. Embryos were fixed for 2 h in a fixative solution (0.2% glutaraldehyde/1% formaldehyde in PBS) at 4°C, washed in PBS, and then stained overnight at 30°C with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining solution [5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl2, 0.02% Nonidet P-40, 0.01% SDS, and 1 mg/ml X-Gal (Boehringer Mannheim)] in PBS. At this point, embryos were paraffin embedded, and sections were stained for LacZ as previously described (1).

Immunofluorescence. In immunofluorescence studies, tissue sections were fixed in acetone bath at 4°C for 10 min, washed twice in PBS, incubated with 0.3% hydrogen peroxide for 10 min at room temperature, and then washed again with PBS. The slides were preincubated in Dako protein block serum-free buffer (DAKO, Carpinteria, CA) for 20 min at room temperature, then incubated for 30 min with a rabbit polyclonal antibody to Escherichia coli β-galactosidase (1:200 dilution) (5 Prime—3 Prime, Inc., Boulder, CO). Sections were then washed with PBS and incubated with a rat monoclonal anti-CD31 antibody (1:200 dilution) (PharMingen, San Diego, CA) for 30 min at room temperature. The sections were washed in PBS and then incubated with a Cy3-conjugated goat anti-rabbit IgG (1:50 dilution; Zymed Laboratories, South San Francisco, CA) and FITC-conjugated goat anti-rat IgG (1:50 dilution; Vector Laboratories, Burlingame, CA) for 30 min at room temperature. After extensive washing in PBS, slides were mounted using DAPI/Antifade (Oncor, Gaithersburg, MD).

RESULTS

Analysis of neonatal and adult Hprt-targeted mice. Tissues were analyzed from F1-F3 progeny of mouse lines derived from two independent ES clones carrying eNOSlaclacZ in the same transcriptional orientation as the Hprt gene and two independent ES cell clones carrying eNOSlaclacZ in the reverse orientation. Since the transgene is subject to X-inactivation in female mice, tissue analysis was confined to male progeny. The patterns and levels of LacZ expression were virtually identical in all four lines of mice. In whole mounts of adult tissues, the X-Gal reaction product was detected in the larger vessels of the heart, spleen, skeletal muscle, brain, kidney, and lungs (Fig. 1, A and B, shows heart and brain, respectively). LacZ staining was more intense on the right side of the heart compared with the left (Fig. 1A). The X-Gal reaction product was present within the aorta and within the blood vessels of the chest wall (Fig. 1C shows chest wall). β-Galactosidase activity was absent in whole mounts of the liver (Fig. 1D).

In cryosections of neonatal and adult tissues, the X-Gal reaction product in the heart was detected in a subset of endothelial cells within the large coronary arteries (Fig. 2A) and myocardial capillaries (Fig. 2B) and within a subpopulation of cardiomyocytes (Fig. 2B). In the coronary arteries, the X-Gal reaction product was present in the endothelial cell lining and to a lesser extent in the smooth muscle cell layer of the arterial wall (not shown). LacZ expression in cardiomyocytes was most pronounced in the right ventricle (not shown). In sections of the brain, β-galactosidase activity was detected in superficial arteries within the subarachnoid and subpial space as well as a small minority of small blood vessels within the inner cortex.
The X-Gal reaction product was detected in occasional large arteries of the lung, skeletal muscle, kidney, and spleen, but was absent in the microvascular bed and veins of these organs (Fig. 2E shows absence of staining in the pulmonary microvasculature). LacZ staining of the liver was consistently negative (Fig. 2F). Finally, β-galactosidase activity within the endothelium was significantly downregulated in adult vs. neonatal mice. In contrast, expression of LacZ in cardiac myocytes was relatively preserved with aging. Immunofluorescent studies proved to be more sensitive than LacZ staining in detecting transgene expression. Using a polyclonal anti-LacZ antibody, we showed more widespread expression of LacZ in the microvessels and endocardium of the heart (Fig. 3) and in arteries of the brain, kidney, spleen, lung, and skeletal muscle (Fig. 4 shows kidney, spleen, and lung). Again, there was no evidence of transgene expression in the vascular bed of the liver (not shown). In contrast to the presence of X-Gal reaction product in the subendothelial lining of the arteries in the heart and brain, immunodetectable LacZ was limited to the endothelial cell lining in these organs.

Analysis of Hprt-targeted embryos. LacZ staining of E10.5 embryos revealed the X-Gal reaction product in the endothelial lining of intersomitic vessels (Fig. 5, A and B), head mesenchyme (Fig. 5C), the aorta (Fig. 5, D–F), and heart (Fig. 5G). In contrast to the presence of endogenous eNOS in blood vessels of the liver, there was no detectable transgene expression in this vascular bed (Fig. 5, H and I).
Fig. 3. Double immunostaining of LacZ and PECAM-1 in cardiac sections from adult Hprt-targeted eNOSlacZ mice. Myocardial sections from two independent lines of mice were double labeled with a rabbit polyclonal LacZ antibody and a rat monoclonal anti-CD31 antibody, washed in PBS, and then incubated with a Cy3-conjugated goat anti-rabbit IgG and FITC-conjugated goat anti-rat IgG. Red color represents LacZ (A and D); green color indicates PECAM-1 (B and E). When superimposed, the two images reveal colocalization of both gene products in microvascular endothelial cells as well as endocardium (C and F).

Fig. 4. Double immunostaining of LacZ and PECAM-1 in noncardiac sections from adult Hprt-targeted eNOSlacZ mice. Tissue sections from adult mice were double labeled with a rabbit polyclonal LacZ antibody and a rat monoclonal anti-CD31 antibody, as described above. Red color represents LacZ (A, D, and G); green color indicates PECAM-1 (B, E, and H). When superimposed, the two images reveal colocalization of both gene products in endothelial cells of a renal artery (C), splenic artery (F), and pulmonary artery (I).
DISCUSSION

The nitric oxide synthases are a family of enzymes that are responsible for generating nitric oxide. eNOS is expressed predominantly in the endothelium where it has been implicated in the control of vasomotor tone as well as vascular remodeling and angiogenesis (14, 21, 23, 24, 27). Despite its designation as a constitutive enzyme, eNOS is upregulated by many extracellular signals, including growth factors, hormones, shear stress and hypoxia (3, 12, 16, 18, 20, 22, 25, 28). It follows that an understanding of its transcriptional control will depend on assay systems that preserve the cell's native microenvironment.

Using standard transgenic approaches with pronuclear microinjection of DNA, we recently showed that the 1,600-bp 5' flanking region of the human eNOS promoter contains information for expression in blood vessels of the heart, skeletal muscle, brain, and aorta (13). In one of the four lines analyzed, reporter gene activity was detected in the pulmonary arteries. The X-Gal reaction product was consistently absent in other vascular beds, such as the spleen and kidney, which otherwise express the endogenous gene product. These results suggested that additional promoter elements either 5' or 3' of the 1,600-bp fragment were necessary for widespread and authentic expression.

In the current study, a single copy of the eNOSlacZ transgene was targeted to the Hprt locus of 129/Ola-derived ES cells. Mice were generated from four independent ES cell clones, with two containing the transgene in the same transcriptional orientation as the Hprt gene and two in the opposite orientation. Analyses of all four lines revealed virtually identical expression patterns. In keeping with the results of the standard transgenic assays (13), reporter gene activity was detected in the endothelial lining of microvessels and macrovessels of the heart and brain and was absent on the venous side of the circulation. However, in contrast to the randomly integrated transgene, the Hprt-targeted construct contained information for expression in a subset of the larger arteries of the kidney, lung, spleen, and skeletal muscle. This expression pattern more closely resembles that of the endogenous eNOS gene. LacZ staining was evident in the adventitia and media of several larger arteries. However, these findings contrasted sharply with the absence of immunodetectable LacZ in the subendothelial layer. The discrepancy between the LacZ staining and immunohistochemistry suggests that the X-Gal reaction product may leak from the endothelium of fixed tissue.

Despite the close correlation between Hprt-targeted eNOSlacZ activity and endogenous eNOS, there was a...
discordance of expression in the vascular bed of the liver. The lack of detectable reporter gene expression in hepatic vessels that otherwise express the endogenous gene suggests that sequences outside the 1,600-bp promoter fragment are necessary for expression in this particular vascular bed. These findings are consistent with those of previous standard transgenic studies (13) and imply that the eNOS gene is regulated by vascular-bed-specific pathways.

The Hprt-targeted eNOSlacZ transgene was also expressed in cardiac myocytes. These cells have recently been shown to contain functional levels of endogenous eNOS (4, 10). Interestingly, the predominance of LacZ-positive cardiomyocytes was located in the right ventricle. This pattern contrasts with the more uniform distribution of the endogenous gene in the heart. It is tempting to speculate that the eNOS gene is regulated in heart muscle by chamber-specific promoter regions. A similar model of cardiomyocyte cell type-specific gene regulation has been invoked in previous studies of the myosin light chain (15, 17). An additional finding in our study was that transgene expression was differentially preserved in cardiomyocytes with aging. The age-related loss of LacZ expression in the endothelium may reflect physiological downregulation of the endogenous gene (5). Alternatively, it may arise from cell type-specific methylation of the promoter-reporter gene cassette. In any case, the findings limit the value of the Hprt-targeted eNOS promoter as a tool with which to target heterologous expression to the vascular bed of the heart in the adult mouse.

The results of the current study suggest that Hprt locus targeting may be employed as a means to study endothelial cell-specific gene regulation. This conclusion is supported by the accompanying report (9), in which the Tie2 promoter was shown to direct cell type specificity at this locus. These studies complement each other in important ways. In this study, we have shown that expression of the Hprt-targeted transgene is highly reproducible between ES cell clones and is independent of orientation. On the basis of these observations, we believe that transgenic analyses may be carried out in single lines of mice. In the companion article, Evans et al. (9) have demonstrated the value of Hprt locus targeting as a means of dissecting the function of a single promoter, in this case the Tie2 gene. When taken together, these two studies provide insight about the differential regulation of two endothelial cell-specific genes. For example, the observation that the Tie2-lacZ transgene but not the eNOSlacZ transgene is expressed in blood vessels of the developing liver and the microcirculation of the lung suggests that the Tie2 promoter alone contains these vascular bed-specific DNA elements.

Another group has recently reported the use of Hprt locus targeting as a means to direct lineage-specific gene expression (26). In this latter study, a 1,565-bp fragment of the myogenin promoter was shown to contain information for correct spatial and temporal expression in embryonic skeletal muscle. However, the targeting strategy employed by these investigators differed in fundamental ways from that used in the present study. First, the myogenin transgene was targeted to a different site in the Hprt locus. Second, the transgene was inserted into AB1 ES cells. Rather than correcting a preexisting Hprt mutation in ES cells, homologous recombination resulted in a disruption of the Hprt gene. Therefore, the cells were not selectable in HAT-supplemented medium. Finally, the myogenin targeting vector contained a PGK-neo cassette. Although the presence of this positive selection cassette did not appear to influence expression of the myogenin promoter, it may potentially affect the regulation of other transgenes that are targeted in a similar fashion.

In summary, the Hprt targeting strategy is a feasible tool for delineating endothelial cell-specific DNA elements in vivo. In more general terms, the Hprt targeting strategy holds promise as a tool for functional genomics. Our findings suggest that other lineage-specific promoters will retain their tissue-specific properties in this locus. If this holds true, then the various promoters may be tested and catalogued according to their sites of expression. As new genes are discovered, they may be tested and catalogued according to their sites of expression. As new genes are discovered, they may be targeted to one promoter or another, targeted to the Hprt locus, and overexpressed in a predictable manner.

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