Regulated Human Erythropoietin Receptor Expression in Mouse Brain*

(Received for publication, July 14, 1997, and in revised form, October 7, 1997)

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Erythropoietin (Epo) is known for its role in erythropoiesis and acts by binding to its receptor (EpoR) on the surface of erythroid progenitors. EpoR activity follows the site of hematopoiesis from the embryonic yolk sac to the fetal liver and then the adult spleen and bone marrow. Expression of EpoR has also been observed in selected cells of non-hematopoietic origin, such as the embryonic mouse brain during mid-gestation, at levels comparable to adult bone marrow. EpoR transcripts in brain decrease during development falling by birth to less than 1–3% of the level in hematopoietic tissue. We have now recapitulated this pattern of expression using a human EpoR transgene consisting of an 80-kb human EpoR genomic fragment. The highest level of expression was observed in the embryonic yolk sac and fetal liver, analogous to the endogenous gene, in addition to expression in adult spleen and bone marrow. Although activity of this transgene in brain is initially lower than the endogenous gene, it does exhibit the down-regulation observed for the endogenous gene in adult brain. The expression pattern of hybrid transgenes of an hEpoR promoter fused to β-galactosidase in 9.5-day embryos suggested that the hEpoR promoter region between −1778 and −150 bp 5’ of the transcription start site is necessary to direct EpoR expression in the neural tube. EpoR expression in the neural tube may be the origin of the EpoR transcripts detected in brain during development. These data demonstrate that both the mouse and human EpoR genes contain regulatory elements to direct significant levels of expression in a developmentally controlled manner in brain and suggest that in addition to its function during erythropoiesis, EpoR may play a role in the development of selected non-hematopoietic tissue.

The erythropoietin receptor (EpoR)† on erythroid progenitor cells is the primary target for erythropoietin (Epo) binding resulting in proliferation and differentiation along the erythroid lineage and is critical for normal erythroid development (1, 2). In hematopoietic cells, the EpoR gene is active early and EpoR mRNA is expressed at moderate levels in the pluripotent hematopoietic stem cell (3). Primary erythroblasts contain a low level of surface erythropoietin receptors, and the number of receptor increase to more than 1000/cell as erythropoiesis continues upon Epo stimulation (4). Late in erythropoiesis, there is a rapid reduction in EpoR expression, and dependence upon Epo declines as cells progress toward terminal differentiation (5). The EpoR gene contains eight exons with a single transmembrane region (6–8) but no intrinsic tyrosine kinase domain (9). Protein phosphorylation and signal transduction via Epo-EpoR stimulation depends in part on activation of JAK2/STAT5 (10, 11) as well as other kinase pathways (12, 13). In vitro, cells of other lineages respond to Epo stimulation including hematopoietic cells such as megakaryocytes, which differentiate in the presence of Epo (14), and B lymphocytes, which exhibit a proliferative response (15).

We have reported that in addition to expression in hematopoietic tissues, EpoR expression can be detected in day 10.5 embryonic mouse brain at high levels comparable to that in adult hematopoietic tissue. EpoR expression in brain decreases with development and is not readily detectable at or after birth (16). Embryonic EpoR expression in the brain may be related to EpoR expression on other non-hematopoietic cells such as those of endothelial or neuronal origin. For example, human umbilical vein endothelial cells express EpoR and are responsive to Epo (17, 18). EpoR mRNA has also been observed in rat brain capillary endothelial cells (19), and functional EpoR has been detected in cultured rodent cells with neuronal characteristics (20, 21) and primary rat hippocampal neurons (22).

In an attempt to create an hEpoR transgenic mouse exhibiting developmental control in both hematopoietic cells and brain, we isolated a larger (80 kb) human genomic fragment containing the EpoR gene for production of transgenic mice. In a preliminary report, we showed that this transgene was able to direct readily detectable levels of hEpoR transgene expression in fetal liver and adult bone marrow with little or no expression in the adult brain (23). We now show that the 80-kb hEpoR transgene is developmentally regulated to mimic both the pattern of the endogenous mEpoR gene expression in hematopoietic tissue and brain. The highest levels of hEpoR transgene and endogenous mEpoR gene expression are observed in hematopoietically active embryonic tissue (yolk sac and fetal liver). In the brain, hEpoR expression in the developing embryo is followed by down-regulation of expression so that the levels of endogenous mEpoR gene and hEpoR transgene expression are reduced about 2 orders of magnitude or more in adult brain. Reporter gene constructs using hEpoR promoter fragments to drive β-galactosidase gene expression in early transgenic mouse embryos suggest that the region flanking the hEpoR proximal promoter is necessary to drive EpoR expression in the early embryonic neural tube. These data demonstrate that both human and mouse EpoR genes are actively transcribed in hematopoietic tissue and the developing embryonic brain and suggest that, in addition to its role in erythropoiesis, EpoR may be functionally important in the development of select non-hematopoietic tissue.

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‡ The abbreviations used are: Epo, erythropoietin; EpoR, erythropoietin receptor; hEpoR, human erythropoietin receptor; mEpoR, mouse erythropoietin receptor; kb, kilobase(s); bp, base pair(s); PCR, polymerase chain reaction; RT, reverse transcriptase.
Fig. 1. A, human EpoR gene fragment used to make transgenic mice. The 15-kb human EpoR DNA fragment contains 2 kb 5′ and 7 kb 3′ of the coding region. The 80-kb hEpoR DNA fragment contains 6 kb 5′ and 60 kb 3′ of the coding region. A hEpoR cDNA probe from exon IV to exon V was constructed to detect the presence of the transgene. B, Southern blot analysis of DNA from normal (Norm) and different transgenic mice (1137, 1139, 1154, and A172). Genomic DNA was digested with BglII and used to confirm the presence of hEpoR after preliminary screening by PCR. M, marker DNA.

MATERIALS AND METHODS

Human EpoR Gene—An 80-kb EpoR genomic clone was isolated from a human P1 phagemic library (Genome Systems, St. Louis, MO). P1 phagemid DNA was prepared for direct injection as described previously (24). DNA was further purified by isopropanol precipitation, ethanol precipitation, and treatment with RNase and phenol/chloroform. Final treatment included Gene-clean (Bio 101, Vista, CA) and filtration through a 0.22-μM filter. The DNA was centrifuged again to remove any remaining glass beads and was dialyzed against injection buffer (10 mM Tris-HCl, pH 7.5, with 0.1 mM EDTA) (16).

Production of Transgenic Mice—Transgenic mice were generated using B6C3F1 (female) × B6D2F1 (male) as described previously (16). The B6C3F1 results from C57BL/6NCr × C3H/HeN and the B6D2F1 results from C57BL/6NCr × DBA/2Ncr. DNA was injected into the male pronucleus of embryonic day 2 (E2) fertilized mouse eggs. The injected eggs were reimplanted into surrogate dams. Screening for hEpoR after preliminary screening by PCR.

Phenylhydrazine Treatment—Normal and transgenic mice were injected with phenylhydrazine at a dose of 0.03 g/g of body weight for two days and at 0.015 g/g of body weight for 3 days. Mice were sacrificed on the 7th day from the initial treatment. The reticulocyte count was determined for each mouse and RNA prepared from different tissues.

EpoR/β-Galactosidase Reporter Gene—A reporter gene construct was made containing a hEpoR promoter fragment extending 1778 bp 5′ of the transcription start site and 3′ to include the untranslated transcribed region and linked to a β-galactosidase reporter gene. Transgenic mice were constructed as described above and embryos harvested and stained for β-galactosidase activity (25). The number of days after vaginal plug formation was used to identify the age of embryos. Embryos were embedded in paraffin and 10-μm sagittal sections were made and counterstained with hematoxylin-eosin.

RESULTS

Transgenic mice were generated from an 80-kb human EpoR clone isolated from a genomic P1 phagemic library. This construct contained flanking genomic DNA extending 6 kb 5′ and 60 kb 3′ of the hEpoR coding region (Fig. 1A). Integration of the transgene was determined by PCR and confirmed by Southern blot analysis as described under “Materials and Methods” (Fig. 1B). Two independent lines of transgenic mice were used for further analysis. At least three mice were analyzed, each independently, from the two lines. Transgene copy number for these lines was low (less than five).

Tissue Distribution of hEpoR Transgene Expression—To examine transgene activity in the adult mouse, RNA was isolated from various tissues and analyzed for endogenous mEpoR gene and hEpoR transgene expression. In hematopoietic tissues, bone marrow, and spleen, both mEpoR and hEpoR transcripts were readily detected (Fig. 2). Expression of the hEpoR transgene in hematopoietic tissue in addition to endogenous mEpoR expression did not result in increased stimulation of erythropoiesis, as no difference was observed in blood hematocrit compared with normal control mice. Mice carrying the 80-kb transgene showed no gross morphological abnormalities. Analysis of expression in non-hematopoietic tissue indicated that no RNA transcripts could be detected in adult liver, heart, leg muscle, or kidney corresponding to either endogenous mEpoR or transgenic hEpoR expression (Fig. 2).

Developmental Expression of the EpoR Transgene in Hematopoietic Tissues—We have previously reported that a 15-kb hEpoR transgene provided hematopoietic specific expression in...
bone marrow was 7.1 fg/mg of RNA. EpoR transcripts in the spleen was determined to be 4.4 fg/µg of RNA for hEpoR and 11.7 fg/µg of RNA for mEpoR. Treatment with phenylhydrazine to induce hemolytic anemia and increase erythropoietic activity resulted in an increase in the percentage of reticulocytes from less than 2% to more than 20% in both normal and transgenic mice. Accompanying the increase in spleen size by 3–4-fold was an increase in endogenous mEpoR gene and hEpoR transgene expression in response to the induced anemic stress (Fig. 4). In contrast, no EpoR expression was observed before or after phenylhydrazine treatment in thymus, a tissue related to lymphoid activity.

Transgene Expression during Brain Development—We have previously reported that in spite of regulated hematopoietic tissue expression, a 15-kb hEpoR transgene was not developmentally regulated in the brain (16). In contrast, the endogenous mEpoR was expressed in embryonic brain tissues at day 10.5 at a level close to that in adult spleen and bone marrow and then decreased with development, dropping by more than 3 orders of magnitude at birth. These data suggested that the hEpoR transgene did not contain regulatory elements to provide control of brain expression and/or that developmental control of EpoR in brain may be different between human and mouse. In the present study, we found that the more distal flanking regions of the hEpoR gene were able to provide regulation in both hematopoietic tissue and brain. An 80-kb human EpoR gene fragment was used to produce transgenic mice; transgenic mice were mated and embryos harvested at different days of development. RNA isolated from brains of 10.5-day embryos exhibited transgene expression of 1.5 fg/µg of RNA (Fig. 5). This level of expression persisted through day 12.5 and then started to decrease with the decrease in endogenous mEpoR expression. Although the 80-kb hEpoR transgene was not able to provide the high level of EpoR expression observed for the endogenous mEpoR gene in the early embryonic (day 10.5) brain, these data suggest that the additional distal flanking genomic sequences provided much of the regulation required for developmental control of brain expression not observed previously.

Human EpoR Promoter Activity in Neural Tube—To determine whether the 5’ DNA region flanking the first exon of hEpoR could direct reporter gene activity in non-hematopoietic tissues in vivo and to identify the possible site of hEpoR expression in the early embryonic brain, the hEpoR promoter linked to a β-galactosidase reporter gene was used to generate transgenic mice. The reporter gene construct contained an hEpoR promoter fragment extending 5’ from the ATG start site for transcription to −1778 bp 5’ of the transcription start site. Mice testing positive for the transgene were mated with normal mice. Embryos were harvested at day 9.5 postcoitum, and whole embryos were stained for β-galactosidase activity. Staining was observed in the ventral neural tube in two independently generated transgenic mouse lines in the region extending from the anterior to posterior tegmental neuropil, the ventral isthmal neuropil, and the pontine neuropil (Fig. 6). However, not all of the transgenic lines exhibited staining at embryonic day 9.5. No staining of the neural tube was observed in normal mice or in transgenic mice containing a shorter promoter that extend to only 150 bp 5’ from the transcription start site (Fig. 6). These data suggest that the promoter region between −1778 and −150 bp is necessary to
drive transgene expression in the neural tube. To confirm that the staining pattern was relevant to EpoR gene expression, tissue corresponding to the β-galactosidase stained region was dissected from normal mice and mice containing the 80-kb hEpoR transgene. Both mEpoR and hEpoR transcripts were detected and quantified by RT-PCR. The levels of hEpoR transgene and mEpoR gene expression in the neural tube were comparable to that in adult hematopoietic tissue and were 5.1 fg/μg of RNA compared with 3.0 fg/μg of RNA for the endogenous mEpoR gene. The expression of EpoR in neural tube at day 9.5 may be the origin of the embryonic brain expression detected later in development.

EpoR Transcripts in Adult Brain—Our initial screening for EpoR expression in the adult brain showed no detectable transcripts, which suggests that if EpoR is expressed, it is at levels less than 0.1 fg/mg of RNA as determined by the quantitative PCR procedure described above. We increased the sensitivity of our analysis and reexamined RNA isolated from normal and transgenic mouse brain tissue. By increasing the PCR cycle number from 30 to 35, we were able to detect mEpoR expression. The PCR product was digested by restriction enzymes XhoI and XmnI and hybridized to mEpoR specific probe to confirm its identity (Fig. 7). This level of expression corresponded to more than 2 orders of magnitude lower than that seen in adult spleen. Comparable results were also obtained for expression in adult brain of the 80-kb hEpoR transgene. As a negative control, increasing the PCR cycle number showed no mEpoR or hEpoR expression in adult liver. No PCR products were observed when reverse transcriptase was omitted from the reaction mixture.

DISCUSSION

The murine system has provided unique insights into the regulation of erythropoiesis by EpoR. Null mutations of both Epo and EpoR gene in mice demonstrated that Epo and EpoR were crucial for definitive erythropoiesis and that interrupted erythroid development in fetal liver was lethal around embryonic day 13 (1, 2). We used the transgenic mouse to examine the developmental regulation of the human EpoR gene. We demonstrated that in transgenic mice produced from the 80-kb hEpoR transgene, appropriate expression of hEpoR was recovered in hematopoietic tissues. The highest level of EpoR expression for the endogenous mEpoR gene and the 80-kb hEpoR transgene occurred in hematopoietic embryonic tissues. The level of EpoR expression in the adult brain of the 80-kb hEpoR transgene. As a negative control, increasing the PCR cycle number showed no mEpoR or hEpoR expression in adult liver. No PCR products were observed when reverse transcriptase was omitted from the reaction mixture.
with development (16). We have now shown that endogenous mEpoR was expressed in the adult brain, but at a level of about 2 orders of magnitude lower than that observed in the embryonic brain at day 10.5. The mEpoR and hEpoR transgene expression we observed in day 9.5 neural tube may be the origin of the EpoR expression detected in the brain at day 10.5 and later and may be related to detection of EpoR localized to the neural plate earlier in development (26). Specific localized binding of radiolabeled Epo to adult mouse brain sections has also been detected (27). These observations suggest that the EpoR message detected in the rodent brain is developmentally regulated and processed into Epo binding protein. The 15-kb hEpoR genomic DNA fragment contained much of the homology between the murine and human EpoR genes within the coding region and extending 5' to the GATA-1 binding site in the EpoR proximal promoter (28, 29). Further 5' are species-specific repetitive elements that have been associated with negative regulation (30–32). However, this human EpoR transgene was expressed at a low level in an unregulated manner in embryonic and adult brain. To include additional regulatory elements, an expanded 80-kb hEpoR genomic fragment was isolated and used to produce transgenic mice.

Analysis of the 80-kb hEpoR transgene demonstrated that embryonic brain expression for both hEpoR and mEpoR decreased in a parallel fashion with further development dropping by about 2 orders of magnitude or more in the adult. The behavior of the hEpoR promoter/β-galactosidase reporter gene constructs suggested that the region from −1778 to −150 bp of the transcription start site was necessary to provide staining of β-galactosidase in the embryonic day 9.5 neural tube. RNA prepared from neural tube of normal and hEpoR transgenic mice at embryonic day 9.5 also showed high level expression of mEpoR and hEpoR and may be the origin of EpoR brain expression observed during development. EpoR expression has been observed in the central nervous system of mid-trimester human fetuses (33) and in brain of adult monkeys (34). GATA-1 is an important transactivator of EpoR expression in hematopoietic cells (28, 29, 35). Although GATA-1 is not expressed in the brain, the brain expresses significant levels of GATA-3, which is capable of binding similar DNA motifs. Targeted disruption of the GATA-3 gene causes severe abnormalities in the embryonic nervous system and in fetal liver hematopoiesis (36). The developmental expression of EpoR expression observed for the endogenous mEpoR gene and the hEpoR transgenes suggests that EpoR expression in non-hematopoietic cells may share some common regulatory controls with hematopoietic expression, but requires additional cis-acting DNA elements.

Although the physiological function in vivo of EpoR expression in non-hematopoietic cells is not yet known, it may be directly related to EpoR activity reported for endothelial cells. Epo stimulation of cultured endothelial cells increases protein phosphorylation and induces nuclear translocation of STAT-5 (37). Epo can also increase intracellular calcium and increase endothelin-1 secretion (38) in a dose-dependent manner (39). The involvement of EpoR in endothelial cells may originate...
from the close relationship between hematopoiesis and vascu-
logenesis (40) and between hematopoiesis and angiogenesis 
later in development (41). Stimulation of EpoR in the brain 
may not require Epo to cross the blood-brain barrier, as oxygen-
dependent Epo production has been observed in cultured rat 
astrocytes (42) and that Epo expression in the adult brain can 
be up-regulated by hypoxia in mice (27) and monkeys (34). 
Expression of EpoR in non-hematopoietic cells may allow for 
Epo activation in the early embryo to stimulate proliferation or 
control apoptosis as those cells differentiate and mature.

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