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Genetics

Polycythemia in transgenic mice expressing the human erythropoietin gene

(gene expression/extramedullary erythropoiesis/pronuclear microinjection/gene dosage)

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ABSTRACT **Erythropoietin is a glycoprotein hormone that regulates mammalian erythropoiesis. To study the expression of the human erythropoietin gene, *EPO*, 4 kilobases of DNA encompassing the gene with 0.4 kilobase of 5′ flanking sequence and 0.7 kilobase of 3′ flanking sequence was microinjected into fertilized mouse eggs. Transgenic mice were generated that are polycythemic, with increased erythrocytic indices in peripheral blood, increased numbers of erythroid precursors in hematopoietic tissue, and increased serum erythropoietin levels. Transgenic homozygotes show a greater degree of polycythemia than do heterozygotes as well as striking extramedullary erythropoiesis. Human erythropoietin RNA was found not only in fetal liver, adult liver, and kidney but also in all other transgenic tissues analyzed. Anemia induced increased human erythropoietin RNA levels in liver but not kidney. These transgenic mice represent a unique model of polycythemia due to increased erythropoietin levels.**

The glycoprotein hormone erythropoietin (EPO) is the primary humoral agent regulating mammalian erythropoiesis (1). EPO gene expression is developmental-stage and tissue-specific, with synthesis of EPO limited to the kidney (2–5), fetal liver (6, 7), and adult liver during stress erythropoiesis (5, 7). EPO gene expression is also inducible, with a several-hundred-fold rise in RNA levels in response to hypoxia (8) or anemia (3–5). Thus, the EPO gene represents an ideal system in which to study regulated gene expression. A rare type of peritubular capillary endothelial cell has been shown to produce EPO RNA in anemic mouse kidney by *in situ* hybridization (4). It has not been possible to establish a pure cell-culture system to study these renal EPO-producing cells *in vitro*. Two human hepatoma cell lines have been shown to express the human EPO gene, with increased EPO RNA levels detected under hypoxic culture conditions (9), but it is not known whether hepatocytes produce EPO *in vivo*.

The human (10, 11) and mouse (12, 13) EPO genes (designated *EPO* and *Epo*, respectively) have been isolated and characterized. Each gene consists of five exons, spanning 2.9 kilobases (kb) of human and 3.4 kb of murine genomic DNA. Nucleotide sequence analysis has revealed a high degree of conservation from 240 base pairs (bp) 5′ of the transcription start site to 220 bp 3′ of the translation-termination codon (12). Sequence conservation is >75% within amino acid coding sequences, 5′ untranslated sequences, 3′ untranslated sequences, and, surprisingly, the first intron of the gene (12). The most highly conserved region of the EPO gene consists of the 140 bp 5′ to the mRNA transcription start site, where there is >90% nucleotide homology and the sequences are very G+C-rich and contain direct repeats; extending 100 bp further 5′ is a region of 53% homology, after which no homology is detected. At the 3′ end of the gene, 53% homology is present within the first 100 bp beyond the termination codon, followed by 120 bp which are 80% homologous and after which there is no detectable homology (12). The sharp demarcation of homology suggested that sequences required for EPO gene expression lie within this region. To test this hypothesis, a 4-kb cloned DNA fragment containing the complete human EPO gene with 0.4 kb of 5′ flanking sequence and 0.7 kb of 3′ flanking sequence was microinjected into fertilized mouse eggs, and transgenic mice carrying the human EPO gene were identified and analyzed.

MATERIALS AND METHODS

Pronuclear Microinjection. A 4-kb *Hind*III–*Eco*RI fragment containing the human EPO gene was subcloned (10) into M13mp9. The insert was purified from vector DNA, bound to glass powder, and resuspended in 10 mM Tris·HCl, pH 7.5/0.25 mM EDTA (14) at a concentration of 1.356 ng/μl, representing 600 copies of the 4-kb DNA fragment per 2 pl. Pronuclear microinjection was performed by using standard techniques (15). (C57BL/6J × A/J) F_1 female mice were superovulated and mated to CD-1 males. Fertilized eggs were recovered, male pronuclei were microinjected, and the eggs were transferred to oviducts of CD-1 pseudopregnant females.

Nucleic Acid Analyses. For identification of transgenic mice, DNA extracted from tails of prepubertal mice was digested with *Bgl* II, followed by gel electrophoresis and blot hybridization (16) to a 0.6-kb *Bst*EII–*Stu* I human EPO cDNA fragment (17), which was ^{32}P-labeled by using random primers (18). For gene dosage analysis, DNA digested with *Kpn* I was blot-hybridized to the human EPO cDNA probe and a 1.4-kb *Dra* I mouse somatostatin gene probe (19).

RNA was isolated from tissues of heterozygous transgenic and nontransgenic littermates by homogenization in guanidinium isothiocyanate and centrifugation through cesium chloride (20). Poly(A)$^+$ RNA (7.5 μg) selected by binding to oligo(dT)-cellulose (21) or total RNA (25 μg) was filtered onto nitrocellulose by using a Minifold II slot-blot apparatus (Schleicher & Schuell) according to the manufacturer's instructions. Filters were hybridized in 50% (vol/vol) formamide/0.75 M NaCl/0.075 M sodium citrate/0.08% Ficoll/0.08% polyvinylpyrrolidone/0.08% bovine serum albumin/0.1% sodium dodecyl sulfate/0.1% sodium pyrophosphate/250 μg of salmon sperm DNA per ml at 42°C (22) to a 0.65-kb *Bgl* II–*Pst* I human EPO gene probe consisting of 3′ untranslated and 3′ flanking sequences. After being washed in 15 mM NaCl/1.5 mM sodium citrate/0.1% sodium dodecyl sulfate at 50°C and autoradiography, the filter was stripped of probe by washing in 5 mM Tris·HCl, pH 8/2 mM EDTA/0.05% sodium pyrophosphate/0.02% Ficoll/0.02% polyvinylpyrrolidone/

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Abbreviations: EPO, erythropoietin; RBC, erythrocyte count.

0.02% bovine serum albumin at 65°C (22) and was rehybridized to a 0.7-kb *Pst* I human β-actin cDNA probe (23).

Anemia was induced by repeated phlebotomy via retroorbital sinus puncture of 12- to 20-week-old transgenic littermates to a final hematocrit of 15–20%, at which time the mice were sacrificed and RNA was isolated. Pregnant female transgenic mice 16–19 days after conception were bled to a hematocrit of 20% for analysis of fetal liver RNA induction.

Hematologic, Radioimmunologic, and Histologic Analyses. Peripheral blood was obtained from male and female F_1 and F_2 littermates at 8–19 weeks of age by retroorbital sinus puncture and analyzed by Coulter Counter in the Clinical Hematology Laboratory of The Johns Hopkins Hospital. Differential cell counts from bone marrow and spleen were expressed as mean values from two experiments in which 8- to 15-week-old transgenic and nontransgenic male littermates were sacrificed; spleen and femoral bone marrow cells were harvested into McCoy's 5A medium, collected by use of Cytospin apparatus (Shandon Southern Instruments), and stained with Wright's stain. Five hundred cells were counted, and the percentage of each cell type was calculated.

EPO levels on pooled serum from 13- to 16-week-old male mice were measured at the SmithKline Bio-Science Laboratories by radioimmunoassay using a polyclonal rabbit antiserum raised against recombinant human EPO (24). Parallelism of normal mouse serum samples with the human EPO standard curve was demonstrated. Sera were pooled from three nontransgenic, four heterozygous transgenic, and five homozygous transgenic mice. The increased number of mice in the transgenic pools was necessary because of their increased hematocrits. Tissues obtained at autopsy were formalin-fixed, blocked in paraffin, sectioned, and analyzed for histologic findings after routine staining with hematoxylin and eosin.

RESULTS

Generation and Genotyping of Transgenic Mice Carrying the Human EPO Gene. A 4-kb DNA fragment containing the complete human EPO gene with 0.4 kb of 5′ flanking sequence and 0.7 kb of 3′ flanking sequence was purified and microinjected into fertilized mouse eggs (Fig. 1*A*). From 320 microinjected eggs transferred to foster mothers, 100 pups were liveborn and 8 carried the transgene, as identified by Southern blot hybridization (16). A mouse designated Tg7 contained one intact copy of the transgene flanked on each side by partial copies in a tandem head-to-tail array (Fig. 1*B*). Tg7 was mated to CD-1 females to generate F_1 transgenic mice, which were then intercrossed (Fig. 1*C*). Heterozygous and homozygous F_2 transgenic mice were identified by gene dosage (Fig. 1*D*). Homozygosity was confirmed by mating homozygotes to nontransgenic mice and demonstrating that all offspring were transgenic.

Phenotype of Transgenic Mice Carrying the Human EPO Gene. Mice of the Tg7 line were next analyzed for physiologic effects of transgene expression. The transgenic mice had significant increases of erythrocyte count (RBC), hematocrit, and hemoglobin levels (Table 1). Therefore, they exhibit polycythemia or erythrocytosis, a disorder characterized by expansion of erythroid mass. For each of the hematologic indices, mean values progressively increased as transgene dosage increased from 0 (nontransgenic, −/−) to 1 (heterozygous transgenic, +/−) to 2 (homozygous transgenic, +/+), with $P < 0.001$ for all pair-wise comparisons between

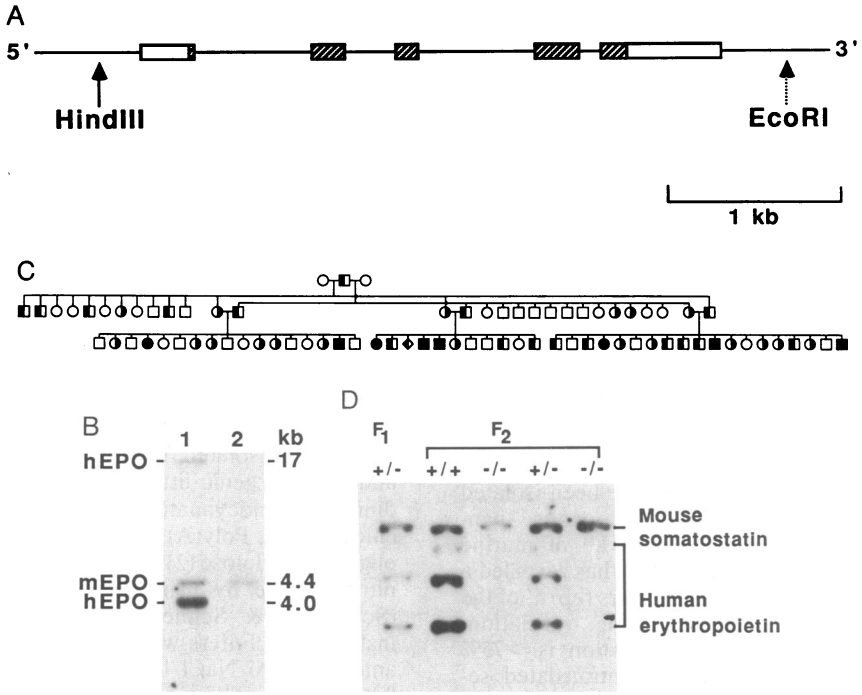

FIG. 1. Generation of the Tg7 transgenic mouse line carrying the human EPO gene. (*A*) Structure of human EPO gene fragment isolated for microinjection. Hatched boxes, translated sequences; open boxes, untranslated sequences; horizontal lines, flanking and intervening sequences; dotted arrow, *Eco*RI site created in cloning not present in genomic DNA (10). (*B*) Analysis of tail DNA for the human EPO (hEPO) gene. Lanes: 1, transgenic mouse Tg7; 2, nontransgenic littermate. DNA extracted from tails of prepubertal mice was digested with *Bgl* II, followed by gel electrophoresis and blot hybridization (16) to a human EPO cDNA probe. Digestion of Tg7 DNA with *Bgl* II, which cuts once within the injected fragment, generates a 4.0-kb unit-size human EPO gene fragment (indicating tandem head-to-tail transgene integration) and a 17-kb junction fragment; a 4.4-kb mouse EPO (mEPO) gene fragment is also seen. (*C*) Pedigree of the Tg7 line. Circle, female; square, male; diamond, stillborn; open symbol, nontransgenic; half-closed symbol, heterozygous transgenic; closed symbol, homozygous transgenic. (*D*) Human EPO gene dosage in transgenic mice. −/−, Nontransgenic; +/−, heterozygous transgenic; +/+, homozygous transgenic. DNA digested with *Kpn* I was blot-hybridized to a human EPO cDNA probe and a somatostatin gene probe as a control for single-copy mouse DNA. The intensity of the three human EPO transgene fragments enclosed within the bracket was compared to that of the mouse somatostatin gene fragment.

Table 1. Hematologic analyses of nontransgenic (−/−) and of heterozygous (+/−) and homozygous (+/+) transgenic Tg7 mice carrying the human EPO gene

Analysis	Results with mice of specific genotype		
	−/−	+/−	+/+
Peripheral blood analyses			
Erythrocytic indices	(*n* = 27)	(*n* = 33)	(*n* = 9)
RBC, no. × 10^{-3}/mm^3			
Range	7.86–10.32	10.88–14.60	13.04–14.50
Mean ± SD	9.51 ± 0.48	12.50 ± 1.00	13.84 ± 0.50
P	<0.001	<0.001	<0.001
Hematocrit, %			
Range	38.2–49.8	51.6–68.6	64.6–73.8
Mean ± SD	44.7 ± 2.2	60.1 ± 4.8	69.3 ± 2.8
P	<0.001	<0.001	<0.001
Hb, g/dl			
Range	13.0–17.2	17.2–24.6	22.4–25.2
Mean ± SD	15.5 ± 0.7	20.8 ± 1.7	23.7 ± 0.8
P	<0.001	<0.001	<0.001
MCV, fl			
Range	44.4–53.2	45.4–50.6	48.2–52.1
Mean ± SD	47.0 ± 2.0	48.1 ± 1.4	50.1 ± 1.4
P	0.015	0.001	<0.001
MCHb, pg			
Range	15.5–17.1	15.0–17.4	16.7–17.7
Mean ± SD	16.3 ± 0.4	16.7 ± 0.5	17.2 ± 0.4
P	0.002	0.019	<0.001
Reticulocytes	(*n* = 8)	(*n* = 12)	(*n* = 7)
% of total RBC			
Range	2.6–4.1	2.6–5.1	2.5–4.7
Mean ± SD	3.4 ± 0.5	3.6 ± 0.8	3.9 ± 0.8
P	0.540	0.531	0.187
Absolute no., no. × 10^{-3}/mm^3			
Range	257–395	353–650	362–624
Mean ± SD	331 ± 45	458 ± 107	531 ± 89
P	0.006	0.146	<0.001
Nonerythroid elements			
WBC, no. × 10^{-3}/mm^3	(*n* = 27)	(*n* = 33)	(*n* = 9)
Range	3.0–10.4	3.4–8.6	3.6–11.6
Mean ± SD	6.0 ± 2.1	5.6 ± 1.6	6.3 ± 2.4
P	0.370	0.270	0.720
Platelets, no. × 10^{-3}/mm^3)	(*n* = 17)	(*n* = 26)	(*n* = 9)
Range	584–1628	194–1374	740–1473
Mean ± SD	1302 ± 281	933 ± 250	1024 ± 211
P	<0.001	0.330	0.016
Differential cell counts from bone marrow and spleen			
Bone marrow cells			
Polymorphonuclear, %	78.5	52.8	—
Early granulocyte, %	6.1	5.8	—
Erythroid, %	7.8	34.5	—
Megakaryocyte, %	0.7	0.4	—
Lymphocyte, %	6.9	6.5	—
Myeloid/erythroid ratio	15.3	2.0	—
Spleen cells			
Polymorphonuclear, %	6.0	6.5	—
Early granulocyte, %	6.5	6.1	—
Erythroid, %	13.0	31.7	—
Megakaryocyte, %	0.6	0.4	—
Lymphocyte, %	73.9	55.3	—
Myeloid/erythroid ratio	63.5	8.8	—

Pairwise comparison of means was performed by using Student *t* test; *P* values are shown from left to right for nontransgenic vs. heterozygous, heterozygous vs. homozygous transgenic, and nontransgenic vs. homozygous transgenic mice. *n*, Number of mice in a category; MCV, mean corpuscular volume; MCHb, mean corpuscular hemoglobin; WBC, leukocyte count.

genotypes. Sexually mature male transgenic mice had higher mean hematocrits than did female transgenic mice (for 19-week-old F_1 male transgenic mice, 62.1 ± 3.7 vs. 53.9 ± 2.0 for female transgenic mice; $P = 0.018$). The transgenic mice had increased absolute reticulocyte counts (Table 1). Mean corpuscular volume and mean corpuscular hemoglobin values were also significantly positively correlated with transgene dosage. In contrast to erythrocytic indices, there was no significant difference in leukocyte counts (Table 1). Transgenic mice also had decreased platelet counts. In bone marrow and spleen, the percentage of erythroid cells was increased and the myeloid-to-erythroid ratio was decreased (Table 1). Thus, polycythemia in these transgenic mice was evident in peripheral blood and hematopoietic tissue. Radioimmunoassay with a polyclonal rabbit antiserum that reacts with both mouse and human EPO revealed a transgene-dosage effect on serum EPO levels, which increased from 6 milliunits/ml in nontransgenic mice ($n = 3$) to 21 milliunits/ml in heterozygous transgenic mice ($n = 4$) to 32 milliunits/ml in homozygous transgenic mice ($n = 5$).

Polycythemia in these transgenic mice has not been generally associated with premature mortality. Founder Tg7 (hematocrit, 55%) was alive at 19 months; eight of nine homozygotes from the F_2 generation were alive at 12 months. Homozygous transgenic mouse 7.2.17 (hematocrit, 74%) was found dead at an age of 6 months. On autopsy, the chest cavity was occupied by a large mass composed almost entirely of erythropoietic tissue (Fig. 2*a*). Extramedullary erythropoiesis was detected in the liver, within skeletal muscle of the leg, and surrounding the great vessels of the heart, pulmonary blood vessels, and aorta (Fig. 2 *b* and *c*). Cardiovascular changes included cardiac hypertrophy and degeneration of vascular endothelial cells, presumably caused by hemodynamic stress from increased blood viscosity. Heterozygous transgenic mouse 7.1.19 (hematocrit, 53%) was sacrificed at 11 months and showed no evidence of extramedullary erythropoiesis. EPO levels in the heterozygotes may not be sufficiently increased to result in extramedullary erythropoiesis.

Expression of the Human EPO Transgene. To determine the tissue types expressing the human EPO gene, RNA was isolated from tissues of transgenic and nontransgenic littermates. Slot-blot analysis of poly(A)$^+$ RNA using a human EPO-specific DNA probe revealed expression in kidney, fetal liver, and adult liver as well as all other tissues studied from transgenic mice, including brain, spleen (Fig. 3*A*), heart, and lung. No signal was detected in any tissue from nontransgenic mice, despite the presence of equivalent amounts of RNA based on hybridization to a β-actin probe. To determine whether the transgene was inducible, total RNA isolated from tissues of transgenic mice made anemic by bleeding versus nonanemic transgenic mice was hybridized to the human EPO-specific probe and then the β-actin probe (Fig. 3*B*). The transgene was inducible in the liver, as indicated by the great difference in EPO probe hybridization to the RNA from the anemic vs. nonanemic mice, compared to the much smaller difference after hybridization with the β-actin probe. Induction was not seen in the kidney or in any other tissue. Further analysis revealed an inverse correlation between hematocrit and hepatic EPO RNA levels, with progressively more human EPO RNA detected in the livers of transgenic mice with progressively lower hematocrits (data not shown), as has been demonstrated for endogenous mouse EPO gene expression in the kidney (5).

DISCUSSION

We have demonstrated that mice derived from the pronuclear microinjection of fertilized eggs with a DNA fragment containing the complete human EPO gene and a limited amount

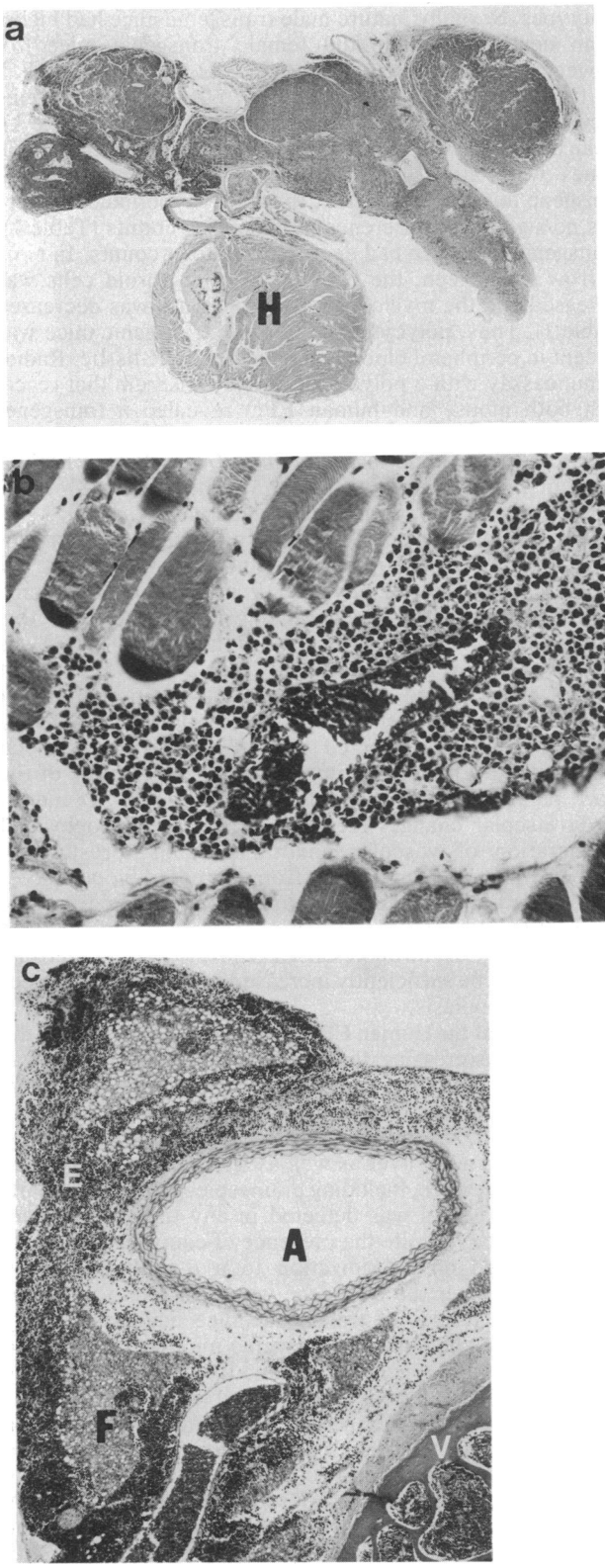

FIG. 2. Extramedullary erythropoiesis in multiple tissues of homozygous transgenic mouse 7.2.17. (*a*) Histologic cross section of heart (H) and thoracic mass composed almost entirely of erythropoietic cells. (×6.3) (*b*) Perivascular infiltration of extramedullary erythropoiesis in striated muscle of leg. (×196.) (*c*) Extramedullary erythropoiesis (E) surrounding the aorta (A). F, paraaortic fat pad; V, vertebra. (×45.5.)

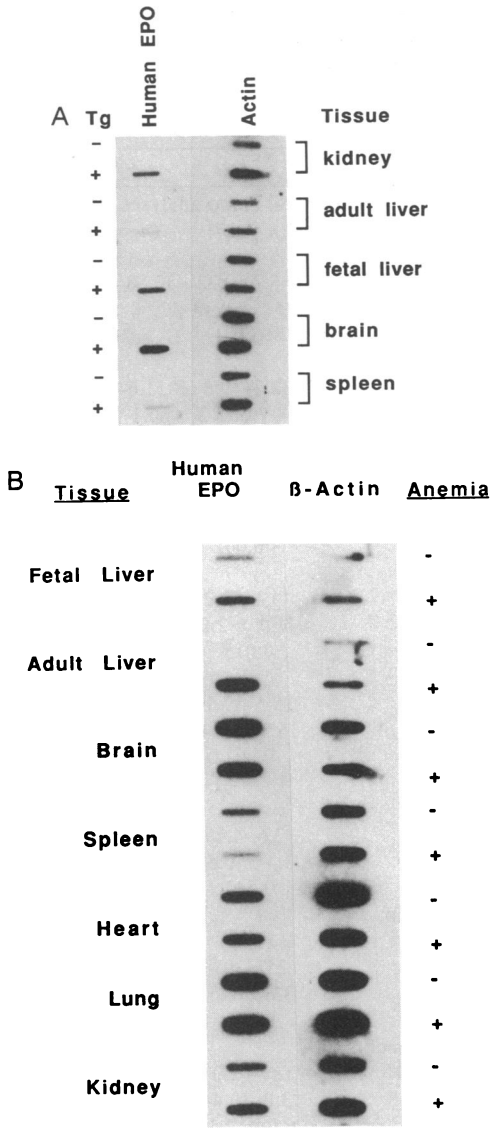

FIG. 3. Slot-blot analysis of human EPO transgene expression. (*A*) Demonstration of human EPO RNA in transgenic (Tg) tissues. Samples (7.5 μg) of poly(A)$^+$ RNA isolated from tissues of transgenic (+) and nontransgenic (−) littermates were filtered in duplicate onto nitrocellulose with a slot-blot apparatus and were hybridized to a human EPO gene-specific probe consisting of 3′ untranslated and 3′ flanking sequences (left lane) and to a β-actin cDNA probe as a control (right lane) for the amount of RNA loaded per slot. (*B*) Induction of human EPO RNA selectively in the liver of anemic transgenic mice. Samples (25 μg) of total RNA isolated from tissues of anemic (+) and nonanemic (−) transgenic mice were hybridized to the human EPO gene-specific probe (left lane). The same filter was then stripped of radioactivity and rehybridized to the β-actin probe (right lane).

of flanking sequences express the transgene and are polycythemic. The transgenic mice not only have increased RBC counts, but the cells are larger and contain more hemoglobin than those of their nontransgenic littermates. The increased percentages of reticulocytes in the peripheral blood of the transgenic mice, although showing a correlation with genotype, are too small to be statistically significant, but the absolute reticulocyte counts, which take into account the increased RBC counts, show differences that, like those of the erythrocytic indices, are highly statistically significant. The polycythemia is also seen in bone marrow and spleen, the hematopoietic tissues of the mouse, where the percentage of erythroid cells is strikingly increased and the myeloid-to-erythroid ratio is correspondingly decreased. A transgene dosage effect was also seen at the level of serum EPO levels,

and the results show that relatively small increases in steady-state EPO levels can cause dramatic expansion of erythroid mass, especially when present over the entire lifetime of an organism. Human EPO RNA is present in the livers of transgenic fetuses (Fig. 3), indicating that human EPO is synthesized prenatally. Thus, these transgenic mice represent a unique model of polycythemia due to increased EPO levels.

The polyclonal radioimmunoassay we utilized measures both mouse and human EPO. Presumably, if the endogenous mouse EPO gene can be down-regulated in the presence of polycythemia, then most, if not all, of the EPO in the transgenic mice may be human. Alternatively, if the endogenous mouse EPO gene maintains a basal level of expression under all circumstances, then the EPO may be both mouse and human in origin. We are attempting to answer this question by developing radioimmunoassays utilizing monoclonal antibodies that react specifically with human EPO versus antibodies that are cross-reacting. The ability of an endogenous gene to down-regulate is of particular importance in the setting of chromosomal trisomy. In the case of Down syndrome, it has been established that certain proteins whose genes map to chromosome 21 show a gene-dosage effect, while others do not (25). It would be of interest to determine whether individuals trisomic for the chromosome 7q11→q22 region (26, 27) to which the human EPO gene has been mapped (28) are polycythemic.

Analysis of RNA from the transgenic mice revealed expression in the tissues in which EPO RNA has been previously detected in humans and mice—the fetal liver, adult liver, and kidney. However, human EPO RNA was also found in all other tissues analyzed, including brain, spleen, heart, and lung. The basal level of transgene expression was also greater than that of the endogenous mouse EPO gene in nontransgenic mice, which is at the limit of detection with a mouse EPO gene probe (3, 4). When EPO RNA was induced by bleeding, increased human EPO RNA was seen in transgenic liver, but not kidney, where the endogenous mouse EPO gene expression increases several-hundred-fold (3, 4). In addition, human EPO RNA expression appears decreased in anemic, as compared with nonanemic, transgenic spleen. While inducible hepatic and noninducible renal transgene expression have been confirmed in multiple subsequent experiments, we have not performed additional studies with spleen RNA to further investigate this phenomenon.

Overall, these results suggest that the 4-kb microinjected DNA fragment contains essential positive regulatory elements that allow high-level human EPO gene expression in transgenic mice but may lack negative regulatory elements, which restrict expression to liver and kidney, as well as positive regulatory elements, which mediate induction in the kidney. To test this hypothesis, additional lines of transgenic mice have been generated. In all three of four lines containing one or more intact copies of the 4-kb human EPO gene fragment, the transgene is expressed, as judged by either the presence of polycythemia or human EPO RNA in various tissues (unpublished data). We have thus defined a DNA fragment of limited sequence that allows expression of the human EPO gene in transgenic mice.

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