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Genetics

Cell-type-specific and hypoxia-inducible expression of the human erythropoietin gene in transgenic mice

(enhancer elements/gene regulation/*in situ* hybridization/polycythemia)

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ABSTRACT **Synthesis of erythropoietin, the primary humoral regulator of erythropoiesis, in liver and kidney is inducible by anemia or hypoxia. Analysis of human erythropoietin gene expression in transgenic mice revealed that sequences located 6–14 kilobases 5′ to the gene direct expression to the kidney, whereas sequences within the immediate 3′-flanking region control hepatocyte-specific expression. Human erythropoietin transcription initiation sites were differentially utilized in liver and kidney. Inducible transgene expression was precisely targeted to peritubular interstitial cells in the renal cortex that synthesize endogenous mouse erythropoietin. These studies demonstrate that multiple erythropoietin gene regulatory elements control cell-type-specific expression and inducibility by a fundamental physiologic stimulus, hypoxia.**

Erythropoietin (EPO) is a glycoprotein hormone that regulates erythrocyte production and, thus, blood oxygen-carrying capacity in mammals. Steady-state EPO RNA levels increase when a rodent is made anemic (1) or hypoxic (2). Hypoxia induces increased EPO RNA levels in Hep3B human hepatoma cells (3, 4), suggesting that oxygen tension is directly sensed by EPO-producing cells. EPO RNA is synthesized in human and murine fetal liver (5, 6), in adult kidney, and, at a lower level, in adult liver (1, 6). Peritubular interstitial cells are the site of EPO synthesis in kidney (7, 8) and the number of EPO-producing cells detected by *in situ* hybridization increases exponentially with decreasing hematocrit (Hct), whereas the intensity of hybridization per cell is unchanged (9).

To identify cis-acting DNA sequences controlling human *EPO* gene expression that is inducible, developmentally regulated, and cell-type-specific, we previously analyzed mice that were transgenic for human DNA encompassing the *EPO* gene and varying flanking sequences. Mice carrying a 4-kilobase (kb) (tgEPO4) or 10-kb (tgEPO10) transgene including the *EPO* gene, 0.7 kb of 3′-flanking sequences, and 0.4 kb or 6 kb of 5′-flanking sequences were polycythemic and showed inducible transgene expression in liver but not in kidney (10, 11). tgEPO10 expression was induced by anemia selectively in hepatocytes surrounding central veins (12). A 256-base-pair (bp) sequence immediately 3′ to the *EPO* gene, which we refer to as the liver inducibility element, bound multiple nuclear factors in gel-shift assays and functioned as a hypoxia-inducible enhancer element in Hep3B transient expression assays (13). In the present study we have generated transgenic mice carrying the human *EPO* gene with more extensive flanking sequences. These transgenic mice express the human *EPO* gene in kidney as well as liver and expression is inducible by anemia and hypoxia.

MATERIALS AND METHODS

Pronuclear Microinjection. Two DNA fragments were prepared for microinjection as follows. (*i*) The complete 18-kb *Eco*RI insert fragment (tgEPO18) of λHEPO3, a bacteriophage clone from a human genomic DNA library (5), was isolated. (*ii*) A library of partially *Mbo* I-digested human lymphoblastoid genomic DNA cloned into cosmid vector pWE-IL2R-Sal (15) was screened with a human EPO cDNA probe from plasmid E49F (5). Recombinant cosmid c59 was plaque-purified and a 22-kb *Eco*RI fragment containing the *EPO* gene (tgEPO22) was isolated. DNA was purified by binding to glass powder, resuspended in 10 mM Tris·HCl, pH 7.5/0.25 mM EDTA at a concentration of 300 copies per pl, and microinjected into the male pronucleus of (C57BL/6 × B6/A)F_1 fertilized mouse eggs that were transferred to the oviducts of pseudopregnant females (10, 11).

Nucleic Acid Analyses. Transgenic mice were identified by DNA blot hybridization and the number of intact transgene copies was determined by gene dosage densitometry (11). Total RNA was isolated (26) and 50 μg was fractionated by 1.4% agarose/2.2 M formaldehyde gel electrophoresis, transferred to a nitrocellulose filter, and hybridized to a nick-translated probe specific for human EPO RNA (11). Seventy-five micrograms of total RNA was hybridized to ^{32}P-labeled antisense RNA probes for 4 hr at 65°C, digested with RNase A and RNase T1, and analyzed by autoradiography after 8 M urea/8% PAGE (11).

Animal Protocols. For Hct determinations, blood was obtained under ether anesthesia by retroorbital sinus puncture. To induce anemia (final Hct = 13–28%), 3- to 9-wk-old F_1 transgenic mice received intraperitoneal injections of 60 μg of phenylhydrazine per g of body weight every 12 hr starting 36 hr prior to sacrifice. To study expression in fetal liver, transgenic males of line 22-43 were mated to nontransgenic females [plug day = day 0 post coitum (p.c.)]. At 14 days p.c., pregnant mice were placed in a chamber maintained at ≈7% O_2 for 4.5 hr and sacrificed, and the fetuses were dissected. Each fetal liver was frozen in liquid N_2 and stored at −80°C. DNA isolated from each fetal carcass was analyzed by blot hybridization to establish presence or absence of tgEPO22. Livers were combined for total RNA isolation from transgenic and nontransgenic pools. Liver RNA from nonhypoxic fetuses was isolated in the same manner. To study postnatal expression, a 5-wk-old mouse of the 18-11 line was bled to Hct = 45%, placed in a chamber maintained at ≈4% O_2 for 5.5 hr, and sacrificed, liver and kidney were frozen in liquid N_2, and total RNA was isolated. Animal protocols were approved by The Johns Hopkins University Animal Care and Use Committee.

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Abbreviations: EPO, erythropoietin; Hct, hematocrit.

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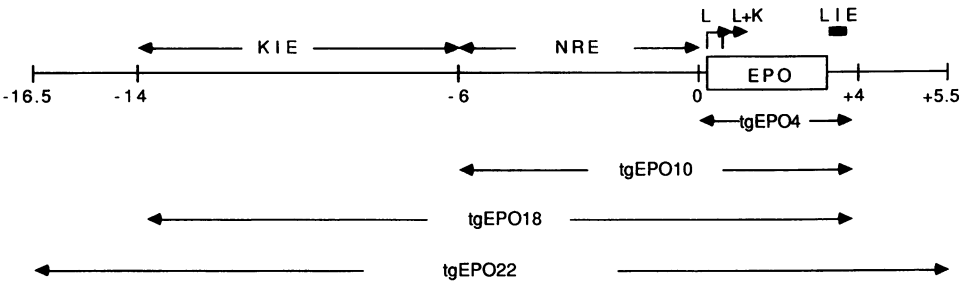


FIG. 1. Human EPO transgenes studied and summary of regulatory regions identified. The top line depicts the human *EPO* gene (box; genomic structure shown in Fig. 3*A*) and surrounding DNA. Below the line are shown coordinates (in kilobases) with respect to the *Hin*dIII site immediately 5′ to the gene (5, 14) and the four EPO transgenes. tgEPO4, tgEPO10, and tgEPO18 are all derived from λHEPO3, isolated from a human genomic bacteriophage library (5), with tgEPO18 representing the complete insert fragment. tgEPO22 is derived from c59, isolated from a human genomic cosmid library (15). Above the line are shown general location of kidney inducibility element (KIE) and negative regulatory element (NRE), the defined liver inducibility element (LIE), and the transcription initiation sites utilized in anemic liver and kidney (L+K) or primarily in liver only (L). The figure summarizes data from refs. 10–13 and this report.

***In Situ* Hybridization.** Paraformaldehyde-fixed, paraffin-imbedded 3-μm kidney sections from anemic mice were hybridized to ^{35}S-labeled antisense RNA probes specific for human or mouse EPO RNA (9, 12).

RESULTS

To identify cis-acting DNA sequences that control human *EPO* gene expression in the kidney, we have generated transgenic mice with extensive EPO flanking sequences: tgEPO18 contains 14 kb of 5′-flanking sequences and 0.7 kb of 3′-flanking sequences, whereas tgEPO22 contains 16.5 kb of 5′-flanking sequences and 2.2 kb of 3′-flanking sequences (Fig. 1). Sixty-four F_0 mice were born following microinjection of tgEPO18 DNA and eight mice (12%) carried one or more intact copies of the transgene (Table 1). Sixty-three F_0 mice were born following microinjection of tgEPO22 DNA, five of which (8%) were transgenic.

Of the eight tgEPO18 F_0 mice identified, seven founders generated F_1 mice for analysis. In six of seven transgenic lines and in the eighth founder, F_0 and/or F_1 mice were polycythemic, with Hct values of 85–87% in the older F_0 mice (Table 1) compared to the nontransgenic mean of 45% and a mean Hct of 56% in tgEPO10 mice, which expressed the transgene only in liver (11). F_1 mice were treated with phenylhydrazine to induce anemia and total RNA from nine organs was analyzed by blot hybridization to a probe specific for human EPO RNA (10, 11). Human EPO RNA was present in kidney and liver total RNA from F_1 mice of six of seven lines tested. Human EPO RNA levels in kidneys of anemic mice were similar to, and in liver greater than, the endogenous mouse EPO RNA levels (data not shown). In the seventh line (18-6), transgenic mice were not polycythemic and did not express human EPO RNA.

Five lines of tgEPO22 mice were also generated (Table 1). Four F_0 transgenic mice were polycythemic, with Hct values of 52–78%. Anemic F_1 transgenic offspring of these founders expressed human EPO RNA in kidney and liver at approximately the same levels as tgEPO18 mice, and human EPO RNA was not detected by blot hybridization in any other tissue (Fig. 2 and Table 1). Since total, rather than poly(A)$^+$-selected, RNA was used we cannot exclude the presence of human EPO RNA in other tissues at levels below the sensitivity of this assay. The size heterogeneity of human EPO RNA in liver compared to kidney is probably due to degradation, as RNase protection assays using this liver RNA preparation resulted in additional smaller fragments for exon 1 and exon 2 (not shown).

The mean Hct values for transgenic mice from three lines (18-46, 18-56, and 22-53) were significantly lower than for the other expressing lines (Table 1). Blot hybridization revealed lower levels of human EPO RNA in kidney and liver of phenylhydrazine-treated transgenic mice from these three lines (data not shown). Thus, there was a correlation between the level of transgene expression in the baseline state (as reflected in the Hct) and the induced state (as detected by blot hybridization). There was no correlation between the number

Table 1. Analysis of human *EPO* expression and its hematologic effects on tgEPO18 and tgEPO22 transgenic mice

Line	DNA copy no.	RNA* Ki	RNA* Li	RNA* O	Hct,† % F_0	Hct,† % F_1	*n*
18-4	10	ND	ND	ND	85	ND	0
18-6	1	−	−	−	ND	45	1
18-11	3	+	+	−	85	84.0 ± 2.7	5
18-39	2	+	+	−	87	84.5 ± 2.4	8
18-44	3	+	+	−	ND	87.0 ± 1.4	2
18-46	4	+	+	−	45	68.7 ± 2.1	3
18-56	4	+	+	−	87	69.4 ± 3.3	5
18-60	3	+	+	−	87	84.0 ± 3.4	4
22-22	1	−	−	−	46	ND	0
22-34	2	+	+	−	64	80.0 ± 7.1	2
22-43	1	+	+	−	78	78.7 ± 1.2	3
22-52	5	+	+	−	52	76	1
22-53	3	+	+	−	61	49.4 ± 2.2	5

ND, not determined.

*Total RNA was isolated from kidney (Ki), liver (Li), and other (O) organs (brain, heart, intestine, lung, spleen, testes, thymus) of 3- to 9-week-old F_1 transgenic mice made anemic by phenylhydrazine treatment, and 50 μg was analyzed by blot hybridization. +, Human EPO RNA detected; −, human EPO RNA not detected.

†Blood was obtained from 3- to 9-month-old F_0 and 2- to 5-month-old F_1 transgenic mice, and Hct was determined by microcapillary centrifugation (mean ± SD shown). *n*, Number of F_1 mice analyzed.

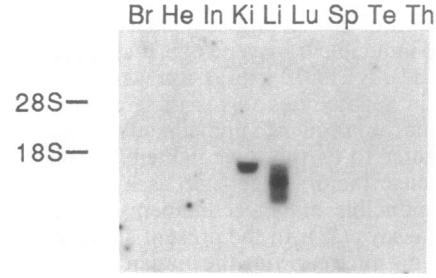
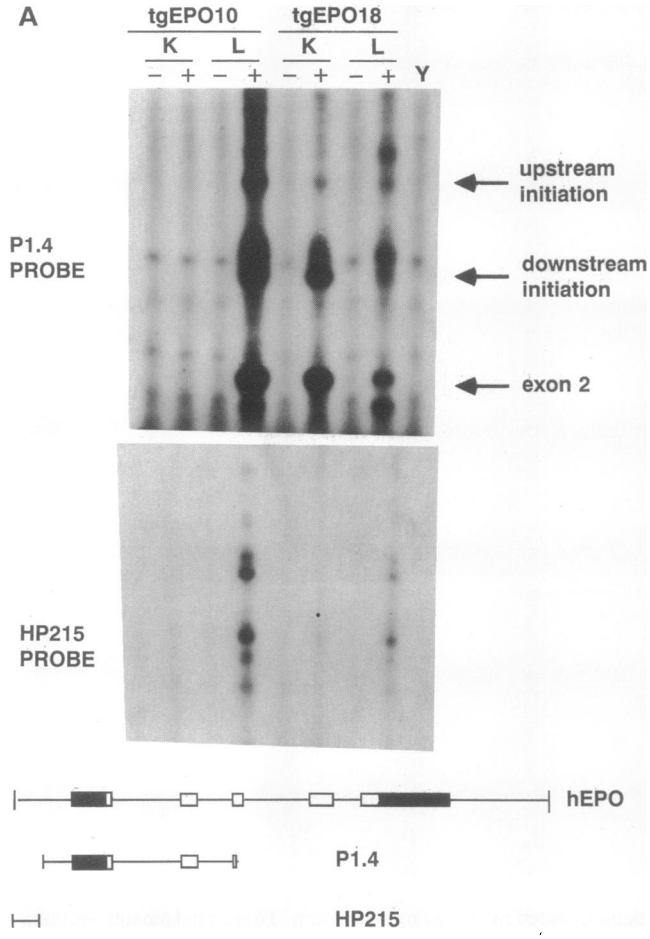


FIG 2. Northern blot analysis of tgEPO22 expression. Fifty micrograms of total RNA isolated from brain (Br), heart (He), intestine (In), kidney (Ki), liver (Li), lung (Lu), spleen (Sp), testes (Te), and thymus (Th) of a transgenic mouse from line 22-43, treated with phenylhydrazine to induce anemia (Hct = 26%), was analyzed by blot hybridization to a probe specific for human EPO RNA. Positions of 18Sand 28S rRNA are indicated.



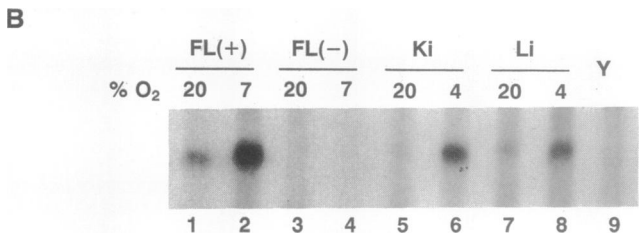


FIG. 3. RNase protection analysis of human EPO RNA in liver and kidney of transgenic mice. (*A*) Differential transcription initiation site utilization and induction by anemia. Total RNA (75 μg) from kidney (K) and liver (L) of tgEPO10 and tgEPO18 mice made anemic (+) or untreated (−) and 75 μg of yeast tRNA (Y) were hybridized to the P1.4 (upper panel) and HP215 (lower panel) ^{32}P-labeled antisense RNA probes at 65°C for 4 hr, digested with RNase A and RNase T1, and analyzed by 8 M urea/8% PAGE. The autoradiograph in the upper panel is of an entire 150-cm slab gel, whereas in the lower panel only part of a 400-cm sequencing gel is shown. The drawing (bottom) depicts the human *EPO* gene (hEPO) and regions transcribed *in vitro* to generate antisense probes. Line, flanking and intervening sequences; closed boxes, untranslated sequences; open boxes, translated sequences. The extent of the 5′-untranslated region in exon 1 is based upon initiation from the downstream site. Upstream initiation sites (not shown on line drawing) map to a region of the HP215 probe (lower panel and ref. 11). (*B*) Expression in fetal liver (FL) and induction by hypoxia. Total liver RNA was isolated from 14-day-gestation transgenic tgEPO22 [FL(+)] and nontransgenic [FL(−)] fetuses carried by mice placed in 7% or 20% O_2 for 4.5 hr prior to sacrifice. Total kidney (Ki) and liver (Li) RNAs were isolated from 5-week-old transgenic tgEPO18 mice placed in 4% or 20% O_2 for 5.5 hr prior to sacrifice. Fifty micrograms of total RNA and 50 μg of yeast tRNA (lanes 1–8) or 75 μg of yeast tRNA only (Y; lane 9) were hybridized to the P1.4 probe and RNase-digested as described in *A*. Only the exon 2 protected fragment is shown.

of copies of tgEPO18 or tgEPO22 and the degree of human *EPO* expression.

To determine the human EPO RNA transcription initiation sites, total RNA from kidney and liver of nonanemic and anemic tgEPO10 and tgEPO18 mice was analyzed by RNase protection assays using two antisense RNA probes (Fig. 3*A*). Probe P1.4 (upper panel) protected the 140-bp exon 2 as well as fragments of 420 bp and 250 bp, which represent exon 1 initiated from the upstream and downstream sites, respectively (11). Probe HP215 (lower panel) protected several fragments representing initiation from the multiple upstream sites (11). Human EPO RNA in the liver of anemic tgEPO10 and tgEPO18 mice was initiated at the upstream and downstream sites, whereas in kidney from tgEPO18 mice, human EPO RNA induced by anemia was preferentially initiated from the downstream site. These data are in agreement with transcript mapping studies of endogenous EPO RNA in mouse kidney (16) and human renal adenocarcinoma cells (17), which demonstrated initiation from the downstream site only. However, primer-extension studies of RNA from Hep3B cells also demonstrated initiation from the downstream site only (17), possibly due to the high GC content of the 5′-flanking region (5). Our studies indicate that human EPO RNA is induced by anemia, and human *EPO* gene transcription initiation sites are differentially utilized in kidney and liver of transgenic mice.

Human EPO RNA levels were also induced by hypoxia. Fourteen-day-gestation tgEPO22 fetuses synthesized human EPO RNA in liver, and transgene expression increased when the mother was placed in 7% O_2 prior to RNA isolation (Fig. 3*B*, lanes 1 and 2). No EPO RNA was detected in liver from nontransgenic sibling fetuses (lanes 3 and 4), demonstrating that the probe is specific for human EPO RNA. Transgene expression was also induced by hypoxia in postnatal liver and kidney (lanes 5–8). Human EPO RNA was detected (at much lower levels) in uninduced kidney (lane 5) and liver (lane 7), suggesting that the greater degree of polycythemia in tgEPO18, compared to tgEPO10, mice is due to the production of human EPO in both kidney and liver in the basal (uninduced) state.

To identify the human EPO-producing cells in kidney, *in situ* hybridization of human and mouse EPO antisense RNA probes (12) to kidney sections from anemic tgEPO10, tgEPO18, and tgEPO22 mice was performed. In each case, endogenous mouse EPO RNA was detected in peritubular interstitial cells in the renal cortex (Fig. 4 *b*, *d*, and *f*) as previously demonstrated (7–9). tgEPO10 mice showed no hybridization to the human EPO probe above background (Fig. 4*a*), but in kidney sections from tgEPO18 and tgEPO22 mice, the same peritubular interstitial cell type that hybridized to the mouse probe also hybridized to the human probe (Fig. 4 *c* and *e*). Furthermore, the number and distribution of cells that hybridized to the mouse and human probes were remarkably similar. In sections of renal cortex from the tgEPO18 mouse, 8070 ± 796 cells per cm^2 (mean ± SD) hybridized to the human EPO probe, compared to 8363 ± 1273 cells per cm^2, which hybridized to the mouse EPO probe (Table 2). In liver, the tgEPO18 and tgEPO22 transgenes were inducibly expressed in perivenous hepatocytes (data not shown), as previously demonstrated for tgEPO10 mice (12). *In situ* hybridization of the human EPO antisense RNA probe to liver sections from hypoxic 14-day-gestation tgEPO22 fetuses (analyzed by RNase protection assays in Fig. 3*B*) demonstrated human EPO RNA within fetal hepatocytes (G.L.S. and S.T.K., unpublished data).

DISCUSSION

Our analyses of mice carrying four different transgenes (tgEPO4, -10, -18, -22), which include the human *EPO* gene

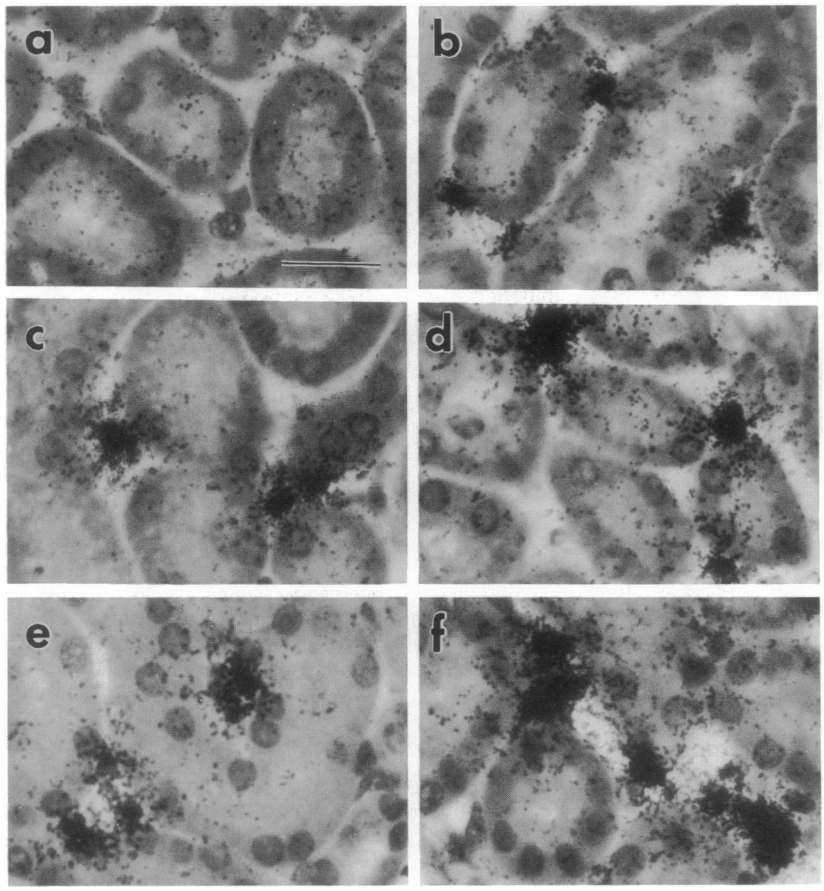


FIG. 4. Cell-type-specific human EPO transgene expression in kidney demonstrated by *in situ* hybridization. Three-micrometer sections of renal cortex from anemic tgEPO10 (*a* and *b*), tgEPO18 (*c* and *d*), and tgEPO22 (*e* and *f*) mice were hybridized to ^{35}S-labeled antisense RNA probes specific for human (*a*, *c*, and *e*) or mouse (*b*, *d*, and *f*) EPO RNA. (Bar = 20 μm.)

with various amounts of flanking DNA, demonstrate that expression of the human *EPO* gene in liver and kidney is controlled by different cis-acting DNA sequences. We previously localized the liver inducibility element to the first 0.3 kb of 3′-flanking sequences (13) and a negative regulatory element, which restricts expression to liver and kidney, to the region 0.4–6 kb 5′ to the gene (11). Inducible expression of tgEPO18, but not tgEPO10, in kidney maps the kidney inducibility element to the region 6–14 kb 5′ to the gene (Fig. 1). The 5′ kidney and 3′ liver elements are thus located on opposite sides of the gene and are at least 10 kb apart. Cis-acting regulatory elements have been identified in the distal 5′-flanking sequences or proximal 3′-flanking sequences of other tissue-specific genes. Mouse albumin (18) and human β-globin (19, 20) gene expression requires sequences 8.5–10.4 kb 5′ and 0.6–0.9 kb 3′ to the gene, respectively. Multiple cis-acting elements controlling expression of a single gene in several different tissues have also been demonstrated. Mouse α-fetoprotein gene expression in yolk sac, fetal liver, and gut is controlled by three enhancer elements located 2.5–6.5 kb 5′ to the gene (21).

Table 2. Enumeration of cells producing human vs. mouse EPO RNA in kidneys of anemic tgEPO18 mice

Parameter	Antisense RNA probe	
	Human EPO	Mouse EPO
Mean (cells per cm^2)	8070	8362
SD	796	1272
Number of sections	8	6
P (Student *t* test)	0.606	

It is not clear whether the 5′ element can function independently of the 3′ element, which was included in each transgene. Kidney nuclear factors bind to the 3′ element, suggesting that the 5′ and 3′ elements may be required for inducible expression in kidney (13). It is clear, however, that the 5′ element precisely targets high-level inducible expression to a specific renal interstitial cell type previously shown to synthesize endogenous mouse EPO. There is no known tissue culture cell line of renal origin that inducibly expresses EPO. Thus it has not been possible to precisely localize the 5′ kidney element by transient expression studies similar to those we have carried out in Hep3B cells to define the hypoxia-inducible 3′ element (13). The targeted expression of a transforming protein such as simian virus 40 large tumor antigen, by inclusion within a chimeric transgene of the *EPO* gene regulatory elements that we have delineated, might

Table 3. Hematocrit vs. EPO production in transgenic mice: Summary and model

Transgene	EPO synthesis*		Hct,† %
	Kidney	Liver	
Non-tg	M	—	45
tgEPO4	M	H	56
tgEPO10	M	H	56
tgEPO18	M + H	H	80
tgEPO22	M + H	H	71

*Baseline EPO RNA synthesis in untreated mice. M, mouse EPO RNA (proposed, based on model of constitutive expression); H, human EPO RNA (demonstrated in Fig. 3 and ref. 11).

†Mean Hct values for lines carrying the same transgene (Table 1 and ref. 11) were used to generate the grand means shown.

result in a tumor of renal EPO-producing cells that could be explanted in tissue culture to establish a permanent cell line, as this strategy has been successfully employed in several other systems (22).

Transcription of the human *EPO* gene is initiated at multiple sites (11) that are differentially utilized in liver and kidney (Fig. 3*A*). The region of the downstream initiation site includes a potential TATA box sequence GATAACA (nucleotides 350–356, ref. 5), whereas the region of the upstream initiation sites includes the extended Sp1 binding site consensus sequence (23) CTCCGCCCA (nucleotides 62–70, ref. 5). Thus, transcription initiation of the human *EPO* gene may be regulated via dual promoters.

tgEPO18 and tgEPO22 mice develop a greater degree of polycythemia than tgEPO4 and tgEPO10 mice, due to human EPO synthesis in kidney as well as liver. RNase protection assays demonstrated the synthesis of human EPO RNA in kidney and liver of untreated transgenic mice (Fig. 3*B*, lanes 5 and 7). Analysis of tgEPO4 and tgEPO10 mice indicated that a negative regulatory element located between 0.4 and 6 kb 5′ to the gene (Fig. 1) prevents expression in tissues other than liver and kidney (11). We propose that baseline *EPO* gene expression in liver and kidney is constitutive (i.e., cannot be extinguished). In adult nontransgenic mice, baseline expression in kidney is sufficient to maintain a Hct of 45%. In tgEPO4 and tgEPO10 mice, human EPO transgene expression in liver results in elevated serum EPO levels (10) and a moderate degree of polycythemia (Table 3). In tgEPO18 and tgEPO22 mice, human EPO transgene expression in kidney and liver results in a greater degree of polycythemia. Based on this model, the presence of polycythemia is not indicative of unregulated expression but rather a gene-dosage effect. To test this hypothesis untreated, polycythemic tgEPO18 mice must be analyzed by *in situ* hybridization for continued production of endogenous mouse EPO RNA in kidney as is predicted by this model.

Humans with chronic renal failure suffer an anemia that is ameliorated by recombinant EPO administration (24), suggesting that the liver cannot compensate for loss of renal EPO production. Expression of all four human EPO transgenes in adult mouse liver is highly inducible by anemia (refs. 10–12; this report), whereas the endogenous mouse *Epo* gene shows only low-level expression (refs. 1 and 12; data not shown). We hypothesized (12) that sequences that repress hepatic *EPO* gene expression in the adult were absent from tgEPO10. A cis-acting element located 0.2–0.8 kb 5′ to the α-fetoprotein gene is responsible for repression of transcription in adult liver (25). Since extensive flanking sequences in tgEPO22 did not affect the magnitude of hepatic transgene expression, if a negative regulatory element does exist, it may be distal to the region encompassed by tgEPO22. These studies demonstrate that utilization of multiple regulatory elements allows the human *EPO* gene to achieve cell-type-specific expression and induction by a fundamental physiologic stimulus, hypoxia.

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