MOLECULAR AND CELLULAR BIOLOGY, Mar. 1990, p. 930–938
0270-7306/90/030930-09$02.00/0

Vol. 10, No. 3

Human Erythropoietin Gene Expression in Transgenic Mice: Multiple Transcription Initiation Sites and *cis*-Acting Regulatory Elements

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Received 20 September 1989/Accepted 29 November 1989

Erythropoietin (EPO) is the primary humoral regulator of mammalian erythropoiesis. The single-copy EPO gene is normally expressed in liver and kidney, and increased transcription is induced by anemia or cobalt chloride administration. To identify *cis*-acting DNA sequences responsible for regulated expression, transgenic mice were generated by microinjection of a 4-kilobase-pair (kb) (tgEPO4) or 10-kb (tgEPO10) cloned DNA fragment containing the human EPO gene, 0.7 kb of 3′-flanking sequence, and either 0.4 or 6 kb of 5′-flanking sequence, respectively. tgEPO4 mice expressed the transgene in liver, where expression was inducible by anemia or cobalt chloride, kidney, where expression was not inducible, and other tissues that do not normally express EPO. Human EPO RNA in tgEPO10 mice was detected only in liver of anemic or cobalt-treated mice. Both tgEPO4 and tgEPO10 mice were polycythemic, demonstrating that the human EPO RNA transcribed in liver is functional. These results suggest that (i) a liver inducibility element maps within 4 kb encompassing the gene, 0.4 kb of 5′-flanking sequence, and 0.7 kb of 3′-flanking sequence; (ii) a negative regulatory element is located between 0.4 and 6 kb 5′ to the gene; and (iii) sequences required for inducible kidney expression are located greater than 6 kb 5′ or 0.7 kb 3′ to the gene. RNase protection analysis revealed that human EPO RNA in anemic transgenic mouse liver and hypoxic human hepatoma cells is initiated from several sites, only a subset of which is utilized in nonanemic transgenic liver and human fetal liver.

Erythropoietin (EPO) is a glycoprotein hormone that plays a principal role in the regulation of mammalian erythropoiesis. EPO gene expression has three major regulatory properties. The first regulatory property is tissue specificity. In rats and mice, EPO RNA is detectable only in liver and kidney (4, 5, 18). Second, EPO gene expression shows developmental stage specificity. In mice, the liver is the major site of synthesis in mid-gestation, after which the kidney plays a progressively greater role in producing EPO (17). Third, EPO gene expression is inducible. Steady-state RNA levels increase several hundredfold in the livers and kidneys of rodents subjected to hypoxia (28), anemia (5), or cobalt chloride administration (4). In adult mice, the basal level of EPO RNA expression is undetectable in liver and at the limits of detection by Northern (RNA) blot hybridization in kidney; after bleeding, EPO RNA is detectable in poly(A)$^+$ liver RNA and total kidney RNA (5). Studies of the human EPO gene have been limited because of low base-line expression and the inability to study inducible expression. EPO RNA has been detected in human fetal liver (16) and in several human hepatoma cell lines under hypoxic culture conditions (12). The kidney is the major site of EPO synthesis in adults, and individuals with chronic renal failure have severe anemia which is ameliorated by recombinant human EPO administration (11).

The single-copy human (16, 21) and mouse (22, 31) EPO genes have been isolated and characterized. Nucleotide sequence homology is >75% within amino acid coding sequences, 5′ untranslated sequences, and 3′ untranslated sequences; 65% within the first intron; and >90% over a 140-base-pair (bp) region 5′ of the transcription start site as mapped in the mouse EPO gene; no significant sequence homology was detected for sequences greater than 250 bp 5′ of the mouse transcription start site or 3′ of the translation termination codon (31).

We produced a line of transgenic mice by microinjection of a 4-kilobase-pair (kb) *Hin*dIII-*Eco*RI genomic DNA fragment containing the intact human EPO gene with 0.4 kb of 5′-flanking sequence (5′-FS) and 0.7 kb of 3′-flanking sequence (3′-FS). Transgenic mice of this line were polycythemic, with increased erythrocytic indices in peripheral blood, increased erythroid precursors in hematopoietic tissue, and increased serum EPO levels compared with nontransgenic littermates (30). Human EPO RNA was detected in liver and kidney but also in all other transgenic tissues tested. Anemia induced increased EPO RNA levels in transgenic liver but not kidney or other tissues. These results suggested that important regulatory elements were absent from the microinjected DNA fragment. To test this hypothesis and to search for additional regulatory elements, we have analyzed four other independently derived lines of transgenic mice carrying the 4-kb transgene and nine lines of transgenic mice carrying an additional 6 kb of 5′-FS. Analysis of these mice revealed that the human EPO gene has multiple transcription initiation sites and *cis*-acting regulatory elements.

MATERIALS AND METHODS

Pronuclear microinjection. A 4-kb *Hin*dIII-*Eco*RI fragment (tgEPO4) was isolated from GEPO 9-2, and a 10-kb *Bam*HI-*Eco*RI fragment (tgEPO10) was isolated from λHEPO3 (16). DNA fragments were purified by restriction endonuclease digestion, agarose gel electrophoresis, and binding to glass powder (15) and suspended in 10 mM Tris hydrochloride (pH

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7.5)–0.25 mM EDTA (8) at 300 copies per pl. Pronuclear microinjection was performed as described previously (15). C57BL/6J × A/J F_1 female mice were superovulated and mated to C57BL/6J males except for transgenic line Tg 7, which was derived by mating to a CD-1 male. Fertilized eggs were recovered, microinjected, and transferred to oviducts of pseudopregnant CD-1 females.

Nucleic acid analyses. DNA was isolated from mouse tail biopsies and analyzed as described previously (30). To determine transgene copy number, 7.5 μg of DNA from F_1 transgenic mice was digested with *Sst*I and analyzed by blot hybridization to a human EPO cDNA probe (29) and to a mouse somatostatin gene probe (24). Autoradiographs were scanned by using an LKB Ultroscan XL laser densitometer. The ratio of EPO to somatostatin band densities was divided by the average of the ratios obtained for Tg 71, 72, and 75 mice, which each carry a single transgene copy.

Total RNA was isolated by guanidine isothiocyanate-phenol-chloroform extraction (9). Poly(A)$^+$ RNA was selected by binding to oligo(dT)-cellulose (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) from 1 mg of total liver RNA and 250 μg of total kidney RNA. Northern blot analysis (32) was performed with 50 μg of total RNA from each tissue, using a nick-translated 0.65-kb *Bgl*II-*Pst*I human EPO genomic DNA fragment (30). After hybridization, blots were washed in 15 mM sodium chloride–1.5 mM sodium citrate at 65°C. To confirm the amount of RNA in each lane, filters were hybridized to a β-actin cDNA probe. To confirm mouse EPO gene induction in kidney, blots were hybridized to a nick-translated 2.4-kb *Sma*I fragment of the mouse EPO gene isolated from plasmid DB2-5 (31).

RNase protection assays (23) were performed by using transcription vectors and reagents from a commercial kit (Promega Biotec, Madison, Wis.) as follows.

(i) pHX1.2. A 1.2-kb *Hin*dIII-*Xba*I human EPO genomic DNA fragment, containing exon 1 with surrounding DNA including 380 nucleotides (nt) 5′ to the putative transcription start site, was subcloned into pGEM4 and digested with *Hin*dIII. Antisense RNA was synthesized by using SP6 RNA polymerase and [α-^{32}P]ATP.

(ii) Pst-1. A 1.4-kb *Pst*I human EPO genomic DNA fragment, containing exons 1 and 2 and surrounding DNA including 160 nt 5′ of the putative start site, was subcloned into pGEM4. *Eco*RI digestion and RNA synthesis by T7 RNA polymerase with [α-^{32}P]ATP generated the probe.

(iii) pHP215. A 215-bp *Hin*dIII-*Pst*I fragment from the 5′-FS was subcloned into pGEM4 and digested with *Hin*dIII. Antisense RNA was synthesized with SP6 polymerase and [α-^{32}P]GTP.

(iv) Pst-6. A 0.85-kb *Pst*I fragment from the 3′ end of the EPO gene was subcloned into pGEM4. *Bgl*II digestion and RNA synthesis by SP6 polymerase with [^{35}S]CTP generated the probe.

RNA probes were DNase I digested, phenol-chloroform extracted, ethanol precipitated, and suspended in 30 μl of hybridization buffer. Hybridizations were performed with 75 μg of total RNA or yeast tRNA and 1 μl of probe in 30 μl of 80% formamide–40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.7)–0.4 M NaCl–1 mM EDTA. Samples were heated to 85°C for 5 min, incubated at 50°C overnight (Pst-6) or 65°C for 3 h (pHX1.2, Pst-1, and pHP215), and then digested with RNase A (40 μg/ml) and RNase T_1 (20 U/ml unless stated otherwise) for 1 h at 37°C, followed by sodium dodecyl sulfate-proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation (23). Samples were analyzed by 8 M urea–5% (or 8% for pHP215) polyacrylamide gel electrophoresis and autoradiography at −80°C for 4 h to 10 days.

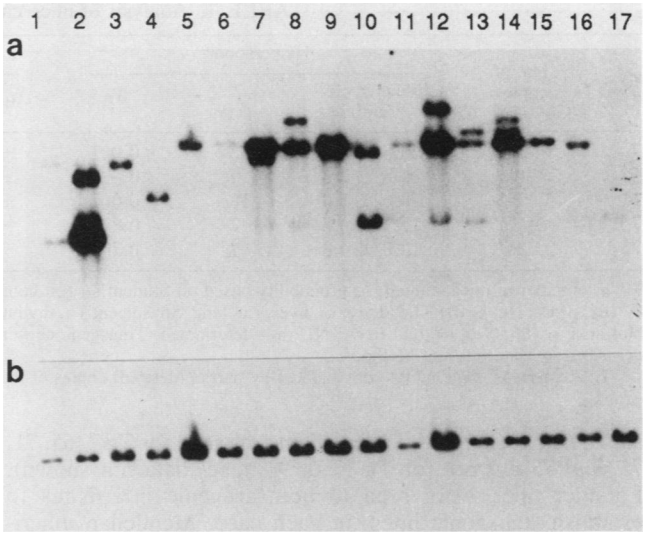

FIG. 1. Analysis of DNA isolated from transgenic mice carrying the human EPO gene. Southern blot analysis of *Sst*I-digested DNA was performed by using a human EPO cDNA probe (a) and a mouse somatostatin gene probe (b). Lanes contain DNA from F_1 transgenic mice of the following lines: 1, Tg7; 2, Tg 65; 3, Tg 71; 4, Tg 72; 5, Tg 75; 6, Tg 98; 7, Tg 102; 8, Tg 117; 9, Tg 118; 10, Tg 126; 11, Tg 132; 12, Tg 137; 13, Tg 163; 14, Tg 165; 15, Tg 167; 16, Tg 176; and 17, nontransgenic mouse. Transgene copy number, as determined by densitometric analysis of autoradiographs, is reported in Tables 1 and 2.

Hematologic analyses. Peripheral blood, collected from mice by retro-orbital sinus puncture, was analyzed by a Coulter counter. Mean hemoglobin values and standard deviations were calculated for each transgenic line and compared with the combined mean (15.1 ± 1.0 g/dl) of 60 nontransgenic mice of the Tg 7, 65, 71, 72, 75, and 98 lines, using Student's *t* test.

Animal protocols. Mice were made anemic by the twice-daily removal of approximately 0.5 ml of blood by retro-orbital sinus puncture under anesthesia and replacement with an equal volume of normal saline by intraperitoneal injection. Animals were sacrificed 6 h after the last bleeding; the final hematocrit was <20% regardless of the initial hematocrit. Cobalt chloride was administered by a single intraperitoneal injection of 60 mg/kg of body weight. Animals were sacrificed by cervical dislocation 6 h after treatment, tissue was harvested immediately into liquid nitrogen, and RNA was prepared. All animal protocols were approved by the Animal Care and Use Committee of The Johns Hopkins University School of Medicine.

RESULTS

Analysis of transgenic mice carrying the 4-kb human EPO transgene. Among 100 liveborn mice derived from fertilized eggs microinjected with the 4-kb *Hin*dIII-*Eco*RI fragment (tgEPO4), 7 contained human EPO DNA: two founders (Tg 13 and 98) carried partially deleted copies of tgEPO4, four founders (Tg 7, 71, 72, and 75) carried a single intact copy, and one founder (Tg 65) carried 30 copies (Fig. 1, lanes 1 to 6; Table 1). To determine whether single transgene copies were intact, DNA was digested with *Fsp*I and *Sst*I, which have sites at nt 143 and 3050, respectively, 3′ to the *Hin*dIII

TABLE 1. Analysis of mice carrying a 4-kb human EPO transgene

Tg	No. of copies	Hemoglobin[a]			Expression of human EPO RNA[b]							
		Mean ± SD (g/dl)	n	P	Br	He	Ki	Li	Lu	Sp	Th	Ind
7	2[c]	20.8 ± 1.7	33	<0.001	+	+	+	+	+	+	ND	+
65	30	20.8 ± 2.6	5	<0.001	+	−	+	+	+	−	+	+
71	1	18.6 ± 1.0	18	<0.001	+	−	+	+	−	−	+	+
72	1	15.1 ± 0.8	7	0.848	−	−	−	+	−	−	−	+
75	1	15.0 ± 0.8	9	0.704	−	−	−	−	−	−	−	−

[a] *n*, Number of mice studied; *P*, probability based on Student's *t* test compared with the nontransgenic mean value of 15.1 ± 1.0.

[b] Br, Brain; He, heart; Ki, kidney; Li, liver; Lu, lung; Sp, spleen; Th, thymus; Ind, inducible expression of transgene in liver; +, present; −, absent by Northern blot analysis of 50 μg of total RNA; ND, not determined. Transgene copy number was determined by densitometric analysis of autoradiographs in Fig. 1 as described in Materials and Methods.

[c] Tg 7 carries a single intact copy flanked by partial (deleted) copies of the transgene.

site. The expected 2.9-kb fragment was seen for Tg 7, 65, 71, 72, and 75 but not for Tg 13 or 98 mice (data not shown). Founder mice were bred to nontransgenic littermates to establish transgenic lines; in each case, Mendelian inheritance of the transgene was observed. Mean hemoglobin values and standard deviations for transgenic mice, compared with the mean for 60 nontransgenic littermates, are shown in Table 1. Tg 7, 65, and 71 mice are polycythemic, with increases in hemoglobin concentration that are statistically significant compared with values for nontransgenic controls ($P < 0.001$, Student's *t* test). A more extensive hematologic analysis of the Tg 7 line has been reported (30).

Total RNA was isolated from brain, heart, kidney, liver, lung, spleen, and thymus from two transgenic mice of each line. One mouse was untreated, and the other was bled to a final hematocrit of 9 to 16%. Total RNA was analyzed by Northern blot hybridization, using a probe specific for human EPO RNA (30). Four of five lines carrying tgEPO4 (Tg 7, 65, 71, and 72) express the transgene (Table 1). In three lines (Tg 7, 65, and 71), the transgenic mice are polycythemic. In all four expressing lines, Northern blot analysis of total RNA from nonanemic mice revealed human EPO RNA in transgenic liver. In three lines (Tg 7, 65, and 71), the transgene was also expressed in kidney and a variable number of other tissues. When total RNA from anemic mice was analyzed, human EPO RNA levels were found to be increased in liver in all four lines but unchanged in kidney. Northern blot analysis using a mouse EPO gene probe confirmed that mouse EPO gene expression was induced in anemic kidney from transgenic mice of each line, and hybridization to a β-actin probe confirmed that equivalent amounts of RNA were present in samples from nonanemic and anemic mice.

Figure 2A shows the increase in human EPO RNA in the liver of anemic Tg 7 and 65 mice. As a positive control, total RNA isolated from Hep3B cells incubated for 24 h in 1% O_2 was also analyzed (Fig. 2a, lane H). These human hepatoma cells have been shown to synthesize a 1.6-kb EPO mRNA under hypoxic culture conditions (12). Human EPO RNA from hypoxic Hep3B cells and from anemic liver of Tg 7 mice comigrate. As a negative control, liver RNA was isolated from Tg 98 mice, which contain a partially deleted copy of the transgene and do not synthesize human EPO RNA, confirming that the probe is specific for human EPO RNA. Human EPO RNA levels in transgenic liver correlated directly with the degree of anemia (Fig. 2b), as is the case for the endogenous mouse EPO gene in the kidney. In tissues other than liver, the transgene was not inducible and the RNA was much larger in size, with the major species migrating above 28S rRNA (Fig. 2b, kidney). This larger RNA was also detected in poly(A)$^+$-selected samples, excluding the possibility of DNA contamination. A Tg 7 mouse was treated with cobalt chloride, and as with anemia, human EPO RNA was increased over the untreated state only in liver (Fig. 2c).

Analysis of transgenic mice carrying the 10-kb human EPO transgene. Among 76 liveborn mice derived from fertilized mouse eggs microinjected with the 10-kb *Bam*HI-*Eco*RI DNA fragment (tgEPO10), human EPO DNA integrated into the genome in 19 mice. Transgenic lines were established from nine different founder mice carrying one to nine copies of the transgene (Fig. 1, lanes 7 to 16; Table 2). Mean blood hemoglobin concentrations for F_1 transgenic mice of each line were compared with the mean value for 60 nontransgenic mice (15.1 g/dl). Transgenic mice of six lines (Tg 118, 132, 137, 163, 167, and 176) were polycythemic, with significantly ($P \leq 0.01$) elevated mean hemoglobin values (16.5 to 26.3 g/dl). In three lines (Tg 102, 117, and 165), the increase in mean hemoglobin (15.8 to 16.3 g/dl) was not statistically significant.

Total RNA was isolated from nine tissues of sex-matched littermate pairs from these lines after bleeding of one member of each pair to a final hematocrit of 12.5 to 19%. Northern blot analysis was performed by using the human-EPO-specific probe. In four lines (Tg 102, 118, 132, and 163), human EPO RNA was detectable only in total liver RNA from anemic transgenic mice (Fig. 2a, lane 118). In four other lines (Tg 137, 165, 167, and 176), human EPO RNA was not detectable in total RNA from any tissue. Because these mice were polycythemic, mRNA from Tg 165 and 167 mice was analyzed. Human EPO mRNA of the appropriate size was detected in poly(A)$^+$ RNA from anemic liver by Northern blot hybridization in each case. Aberrant tgEPO10 expression occurred only in Tg 117 mice, which expressed the transgene in brain, lung, spleen, testes, and thymus but not in liver, nor was the transgene inducible by bleeding. The EPO RNA in Tg 117 mice was larger than 28S rRNA. EPO RNA isolated from anemic liver of Tg 102, 118, 132, and 163 mice was of appropriate size. Administration of cobalt chloride to a Tg 102 mouse also resulted in selective liver induction of transgene expression (not shown).

These results can be summarized as follows. (i) Analysis of total RNA from tgEPO4 mice revealed expression in four of five lines, all four of which showed regulated liver transgene expression and absence of regulated kidney transgene expression; three lines showed inappropriate expression elsewhere. Transgenic mice from three of five lines were polycythemic. (ii) Among nine lines of tgEPO10 mice, analysis of total RNA revealed expression in five lines. In four lines, expression was limited to anemic liver, where the

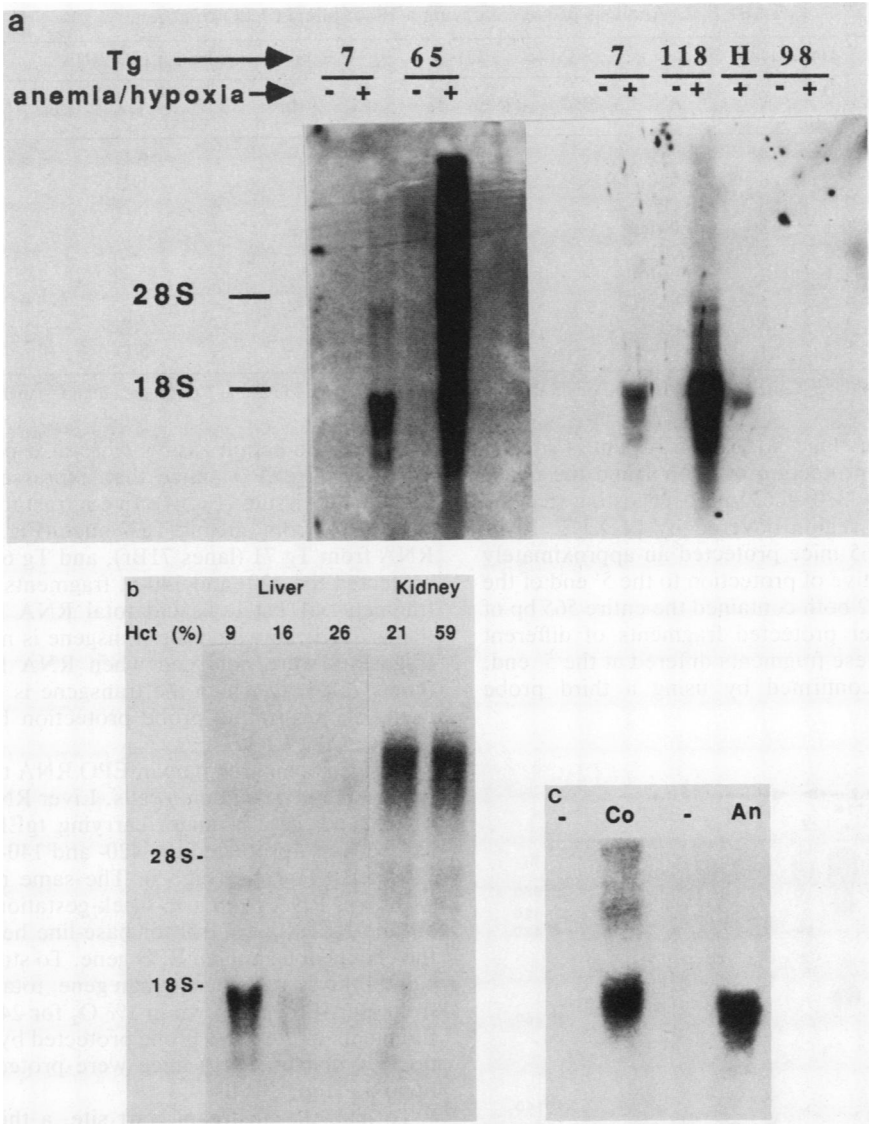

FIG. 2. Analysis of RNA isolated from transgenic mice carrying the human EPO gene. Northern blots containing 50 μg of total RNA per lane were hybridized to a human-EPO-specific probe. Positions of 18S and 28S rRNA are indicated. (a) Induction of human EPO RNA in liver of tgEPO4 and tgEPO10 transgenic mice. Analysis of liver RNA from nonanemic (−) and anemic (+) mice of Tg 7, 65, 98, and 118 lines and of RNA from hypoxic Hep3B cells (H) is shown. The figure is a composite of two autoradiographs; the same Tg 7 RNA samples were analyzed on both, thus allowing for comparison between blots. (b) Comparison of transgene expression in liver and kidney of Tg 7 mice, showing analysis of liver and kidney RNA from mice with indicated hematocrit (Hct). (c) Comparison of human EPO transgene induction in liver by cobalt chloride versus anemia, showing analysis of RNA from untreated (−), cobalt chloride-treated (Co), and anemic (An) Tg 7 mice.

level of expression was greater than that of the endogenous mouse EPO gene, which cannot be detected by using total RNA. In two lines, analysis of poly(A)$^+$ RNA revealed liver expression at a level similar to that of the mouse EPO gene. One tgEPO10 line showed aberrant expression and absence of liver inducibility. Transgenic mice from six of nine lines were polycythemic. (iii) None of the tgEPO4 or tgEPO10 transgenic mice showed regulated transgene expression in kidney. (iv) Hepatic expression of tgEPO4 and tgEPO10 was inducible by both anemia and cobalt chloride administration.

Analysis of the initiation of human EPO gene transcription. RNase protection assays were performed by using antisense RNA probes from the 5′ end of the human EPO gene. Total RNA was hybridized to ^{32}P-labeled antisense RNA at 65°C for 3 h and RNase digested, and protected fragments were sized by denaturing polyacrylamide gel electrophoresis. Figure 3 shows the results of analysis of liver RNA from nonanemic and anemic Tg 65 and Tg 118 mice carrying the tgEPO4 and tgEPO10 transgenes, respectively. Human EPO RNA was greatly increased in liver from anemic compared with nonanemic transgenic mice. Fragments of approximately 140 and 250 nt were protected when the Pst-1 probe (Fig. 3a) was hybridized to RNA from anemic liver. However, a 410 to 420-nt fragment was also detected, consistent with protection from the 3′ end of exon 1 to the 5′ end of the probe (250 + 160 nt), as a result of initiation 5′ to the expected start site. This hypothesis was supported by nuclease protection assays using the pHX1.2 probe with more extensive 5′-FS. Liver RNA from anemic Tg 118 mice protected a 250-nt fragment of pHX1.2, as expected, and a fragment which, given limited resolution at the top of the gel, sized to 475 to 540 nt on various gels (mean ± standard

TABLE 2. Analysis of mice carrying a 10-kb human EPO transgene

Tg	No. of copies	Hemoglobin			Expression of human EPO RNA[a]										
		Mean ± SD (g/dl)	n	P	Br	He	In	Ki	Li	Lu	Ov	Sp	Te	Th	Ind
102	7	15.8 ± 0.5	4	0.179	−	−	−	−	−	−	ND	−	−	−	+
117	4	15.9 ± 0.9	8	0.060	+	−	−	−	−	+	ND	+	+	+	−
118	6	17.2 ± 1.0	9	<0.001	−	−	−	−	−	−	ND	−	−	−	+
132	1	26.3 ± 0.1	2	<0.001	−	−	−	−	−	−	−	−	ND	−	+
137	6	16.7 ± 1.3	6	0.001	−	−	−	−	−	−	−	−	ND	−	−
163	2	25.5 ± 1.1	3	<0.001	−	−	−	−	−	−	−	−	ND	−	+
165	9	16.3 ± 1.9	3	0.063	−	−	−	−	−	−	−	−	ND	−	−
167	1	16.5 ± 1.7	5	0.008	−	−	−	−	−	−	ND	−	−	−	−
176	1	16.5 ± 0.4	4	0.010	−	−	−	−	−	−	ND	−	−	−	−

[a] In, Intestine; Ov, ovary/oviduct; Te, testes. Methods and other abbreviations are as explained in Table 1 (footnotes *a* and *b*) and the text.

deviation = 517 ± 25 nt; Fig. 3a). This fragment is smaller than that expected for protection of exon 1 and the entire 5′-FS in pHX1.2 (250 + 380 = 630 nt), suggesting that the start site is within the region covered by pHX1.2. Liver RNA from anemic Tg 65 mice protected an approximately 680-nt fragment, suggestive of protection to the 5′ end of the probe. Pst-1 and pHX1.2 both contained the entire 565 bp of intron 1 but gave larger protected fragments of different sizes, suggesting that these fragments differed at the 5′ end. This hypothesis was confirmed by using a third probe described below.

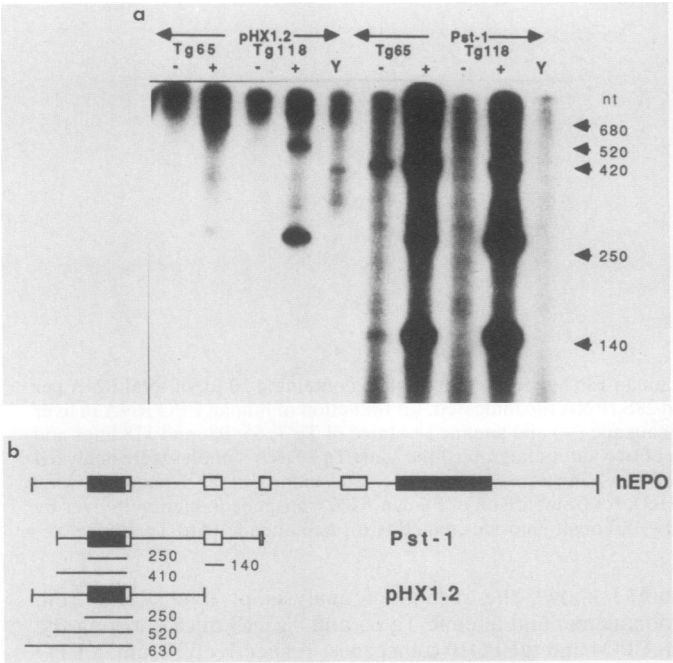

FIG. 3. RNase protection analysis of liver RNA from transgenic mice. (a) Analysis of 75 μg of total RNA from nonanemic (−) and anemic (+) Tg 65 and Tg 118 transgenic mice and 75 μg of yeast tRNA (Y) hybridized to pHX1.2 (left) and Pst-1 (right) antisense RNA probes. The preparations were RNase digested, and products were analyzed by denaturing polyacrylamide gel electrophoresis and autoradiography. (b) Map of the human EPO gene (hEPO) and probes. Symbols: ■, untranslated sequences; □, amino acid-coding sequences; ——, intervening and flanking sequences. Sizes (in nucleotides) of protected fragments, determined relative to molecular size markers on the gel (right side of panel a), can be compared with sizes predicted by known sequence (b); extent of 5′ untranslated sequence is based on homology to the mouse EPO gene (described in the text).

RNase protection assays were also performed with total RNA from tgEPO4 mice that expressed the transgene in nonhepatic tissues (Fig. 4). In contrast to the pattern seen in liver RNA from anemic Tg 7 mice (Fig. 4, lane 7Li), brain RNA from Tg 71 (lanes 71Br), and Tg 65 (not shown) mice protected the 420- and 140-nt fragments, but not the 250-nt fragment, of Pst-1, as did total RNA from Tg 71 kidney (lanes 71Ki), in which the transgene is not inducible. These fragments were not seen when RNA from Tg 72 kidney (lanes 72Ki), in which the transgene is not expressed, was used, demonstrating probe protection by human, and not mouse, EPO RNA.

We also compared human EPO RNA transcribed in transgenic mice versus human cells. Liver RNA from nonanemic Tg 65 and Tg 118 mice, carrying tgEPO4 and tgEPO10, respectively, protected the 420- and 140-nt fragments of the 1.4-kb Pst-1 probe (Fig. 5). The same result was obtained with liver RNA from a 16-week-gestation human fetus (Fig. 5, lane HFL), a control for base-line hepatic expression of the endogenous human EPO gene. To study induced expression of the endogenous human gene, total RNA was isolated from Hep3B cells grown in 1% O_2 for 24 h. The same three fragments of the Pst-1 probe protected by RNA from anemic tgEPO4 and tgEPO10 mice were protected by RNA from hypoxic Hep3B cells.

To map the upstream start site, a third probe, pHP215,

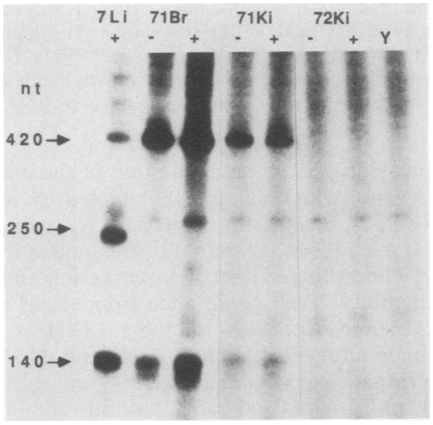

FIG. 4. RNase protection of the Pst-1 probe by RNA from tgEPO4 transgenic mice. Sources of RNA analyzed: liver of anemic (+) Tg 7 mouse (7Li); brain of nonanemic (−) and anemic Tg 71 mice (71Br); kidney of nonanemic and anemic Tg 71 (71Ki) and Tg 72 mice (72Ki); Y, yeast tRNA. Sizes of protected fragments are indicated on the left.

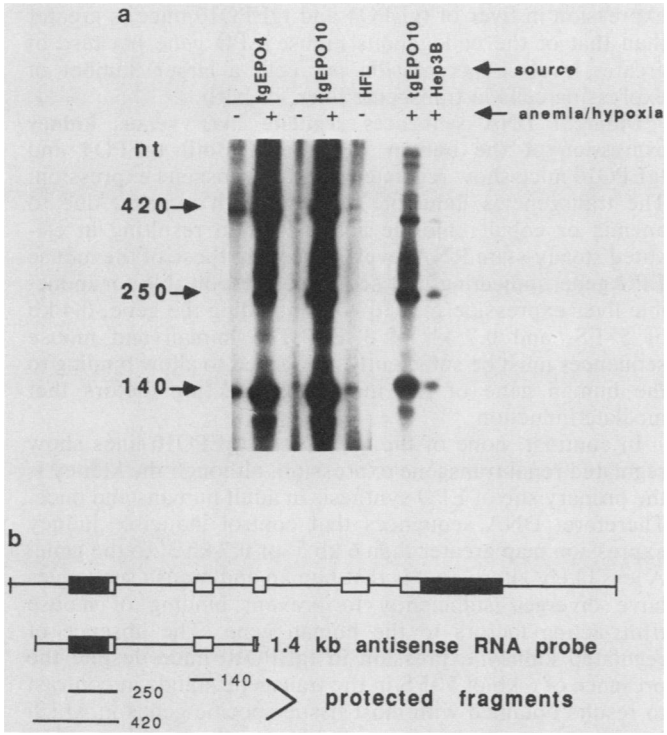

FIG. 5. Protection of the Pst-1 probe by RNA from transgenic mice and human cells. (a) Analysis of 75 μg of total RNA from liver of nonanemic (−) and anemic (+) Tg 65 and Tg 118 mice carrying tgEPO4 and tgEPO10, respectively; human fetal liver (HFL); and hypoxic Hep3B cells. Sizes (in nucleotides) of protected fragments is shown on the left. (b) Map of the human EPO gene (top line), Pst-1 (1.4-kb antisense RNA) probe, and protected fragments.

which contains 215 nt at the 5′ end of pHX1.2 and has no sequences in common with RNA initiated at the downstream site, was used (Fig. 6). When pHP215 was hybridized to liver RNA from anemic tgEPO10 mice, 72-, 82-, 88-, 100-, 105-, and 118-nt fragments of the probe were protected. The same fragments were protected by total RNA from hypoxic Hep3B cells. Full-length probe (215 nt) was protected by liver RNA from anemic tgEPO4 but not tgEPO10 mice (not shown). No protection occurred with liver RNA from Tg 98 mice, carrying a deleted transgene copy.

These results can be summarized as follows. (i) Two sets of initiation sites are utilized in transcription of the human EPO gene in transgenic mice and human cells. (ii) Base-line transcription is initiated from several upstream sites, generating EPO RNA species with an additional several hundred nucleotides at their 5′ ends. (iii) After induction by anemia or hypoxia, increased transcription is initiated from the upstream sites and from the downstream site.

Analysis of human EPO RNA polyadenylation. The human EPO RNA polyadenylation site was mapped in cDNA clones with poly(A) tracts 11 to 13 nt downstream from the sequence AAGAAC (16, 21). The 3′ end of human EPO RNA in transgenic mice was mapped by using the Pst-6 probe (Fig. 7). Human EPO RNA ending at the site predicted by cDNA would protect 380 nt of the probe. The predicted fragment was protected from digestion by liver RNA from anemic Tg 7, 65, 118, and 132 mice, whereas no protection was seen with liver RNA from anemic Tg 98 mice containing a partially deleted transgene, liver RNA from nonanemic mice, and kidney RNA from nonanemic or anemic mice. Human EPO RNA synthesized in liver of tgEPO4 and tgEPO10 mice is therefore correctly polyadenylated.

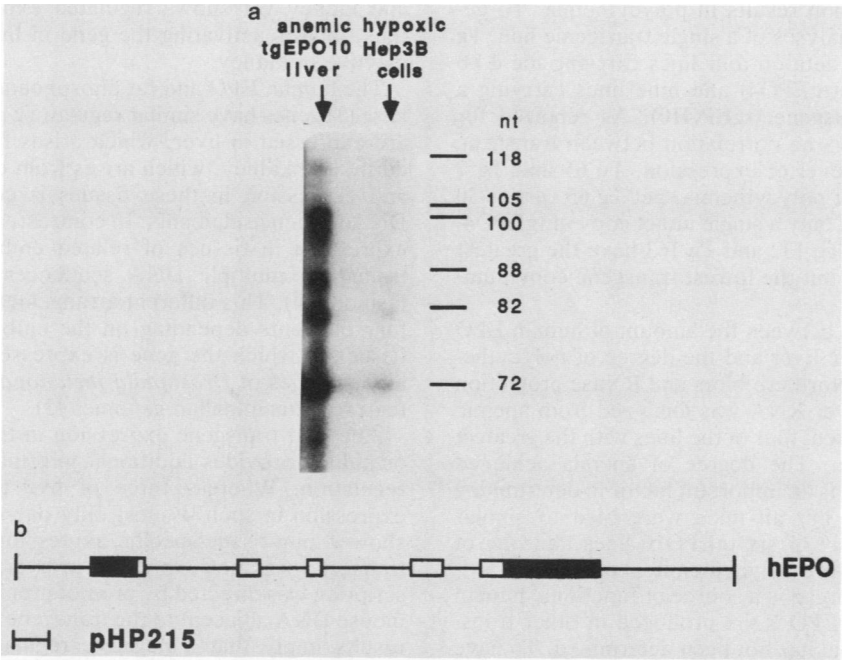

FIG. 6. RNase protection of the pHP215 probe by RNA from transgenic mice and human cells. (a) Analysis of 75 μg of total RNA from liver of anemic Tg 118 mice carrying tgEPO10 and from hypoxic Hep3B cells. Sizes of protected fragments are shown on the right. (b) Map of the human EPO gene (hEPO) and pHP215 probe. As in Fig. 3 to 5, extent of 5′ untranslated sequence is based on homology to the mouse EPO gene.

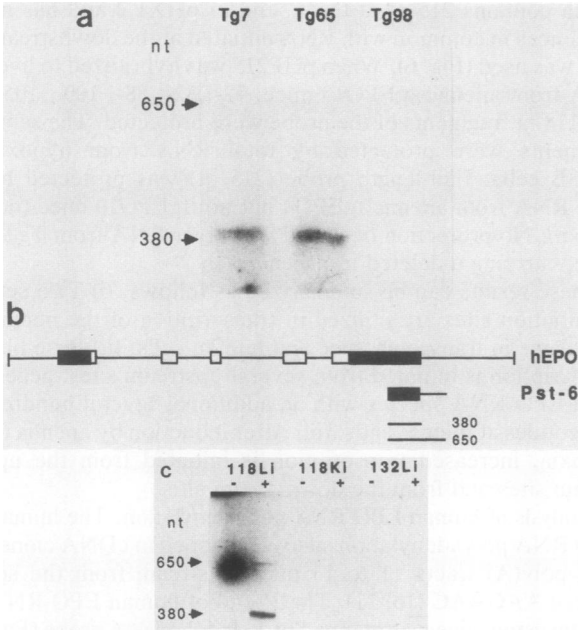

FIG. 7. Mapping of the polyadenylation site in human EPO RNA from transgenic mice, using the Pst-6 probe. (a) Analysis of 75 μg of total RNA from liver of anemic Tg 7, Tg 65, and Tg 98 mice. Sizes of protected fragments are shown on the left. (b) Map of the human EPO (hEPO), Pst-6 probe, and protected fragments (sizes in nucleotides). (c) Analysis of total RNA from liver (118Li and 132 Li) and kidney (118Ki) of nonanemic (−) and anemic (+) Tg 118 and Tg 132 mice.

DISCUSSION

Phenotype of transgenic mice expressing the human EPO gene. Transgenic mice carrying the human EPO gene with 0.4 kb of 5′-FS and 0.7 kb of 3′-FS express the gene, and increased EPO production results in polycythemia. To genetic and physiologic analyses of a single transgenic line, Tg 7 (30), we now present data on four lines carrying the 4-kb human EPO transgene (tgEPO4) and nine lines carrying a 10-kb human EPO transgene (tgEPO10). As reported for other genes (25), there is no correlation between transgene copy number and the level of expression. Tg 65 and Tg 7 have the same degree of polycythemia, yet Tg 65 carries 30 copies whereas Tg 7 has only a single intact copy of tgEPO4. Among tgEPO10 lines, Tg 132 and Tg 163 have the greatest degree of polycythemia but the lowest transgene copy number.

There is a correlation between the amount of human EPO RNA detected in anemic liver and the degree of polycythemia. Strong signals on Northern blots and RNase protection gels were seen when liver RNA was analyzed from anemic Tg 7, 65, 118, and 132 mice, four of the lines with the greatest degree of polycythemia. The degree of anemia achieved before isolation of RNA is an important factor in determining EPO gene expression, but all mice were bled to similar hematocrits. Because six of six tgEPO10 lines and one of three tgEPO4 lines that are polycythemic express the transgene only in liver, this organ is a source of functional human EPO mRNA. Whether EPO RNA produced in other transgenic tissues is functional has not been determined. To have a physiologic effect, EPO RNA is transcribed, processed, and translated, and the protein is glycosylated and secreted into the circulation. EPO RNA produced in nonhepatic tissues may not fulfill these requirements. Human EPO RNA expression in liver of tgEPO4 and tgEPO10 mice is greater than that of the endogenous mouse EPO gene because of greater levels of expression per cell, a larger number of expressing cells in transgenic liver, or both.

Different DNA sequences regulate liver versus kidney expression of the human EPO gene. Both tgEPO4 and tgEPO10 mice show regulated hepatic transgene expression. The transgene is inducible in liver, with hypoxia due to anemia or cobalt chloride administration resulting in elevated steady-state RNA levels exceeding those of the mouse EPO gene, indicating that sequences responsible for inducible liver expression map to 4 kb including the gene, 0.4 kb of 5′-FS, and 0.7 kb of 3′-FS. The human and mouse sequences must be sufficiently conserved to allow binding to the human gene of the mouse *trans*-acting factors that mediate induction.

In contrast, none of the tgEPO4 or tgEPO10 lines show regulated renal transgene expression, although the kidney is the primary site of EPO synthesis in adult humans and mice. Therefore, DNA sequences that control inducible kidney expression map greater than 6 kb 5′ or 0.7 kb 3′ to the gene. A less likely alternative is that human and mouse sequences have diverged sufficiently to prevent binding of mouse *trans*-acting factors to the human gene. The absence of regulated kidney expression in tgEPO10 mice despite the presence of 6 kb of 5′-FS in the transgene stands in contrast to results obtained with most tissue-specific genes in which regulatory sequences map to the first several kilobase pairs of 5′-FS (25). The linked mouse alpha-fetoprotein and albumin genes are exceptions in which tissue-specific enhancers are located 2.5 to 6.5 kb (14) and 8.5 to 10.4 kb (26), respectively, 5′ to the gene, as is the human β-globin gene, which requires sequences 0.6 to 0.8 kb 3′ to the poly(A) site for tissue-specific expression (2). Regulation of liver and kidney EPO gene expression by different DNA sequences implies that different *trans*-acting factors interact with the gene in these tissues. The transgene is present in both liver and kidney yet shows regulated expression only in liver; thus, factors activating the gene in liver must be absent or inactive in kidney.

The human EPO and rat phosphoenolpyruvate carboxykinase (3) genes have similar regulatory properties. Both genes are expressed in liver, which arises from embryonic endoderm, and kidney, which arises from embryonic mesoderm, and expression in these tissues is controlled by different DNA sequence elements. In contrast, alpha-fetoprotein gene expression in tissues of related embryonic origin is controlled by multiple DNA sequences utilized in a shared fashion (14). This different arrangement of *cis*-acting regulatory elements depending on the embryonic relationship of tissues in which the gene is expressed, originally detected among genes of *Drosophila melanogaster* may be a general feature of mammalian genomes (3).

Aberrant transgene expression in tissues other than liver or kidney provides additional information about EPO gene regulation. Whereas three of five tgEPO4 lines showed expression in such tissues, only one of nine tgEPO10 lines showed non-tissue-specific expression, and in this line the transgene was not expressed in liver, suggesting that transcription was directed by promoter or enhancer sequences in mouse DNA adjacent to the transgene integration site. These results imply that a negative regulatory element, located between 0.4 and 6 kb 5′ to the gene, restricts expression to liver and, presumably, kidney. Negative regulatory elements have been detected in many eucaryotic genes (1, 10, 13, 19, 20), and the presence of multiple positive and negative

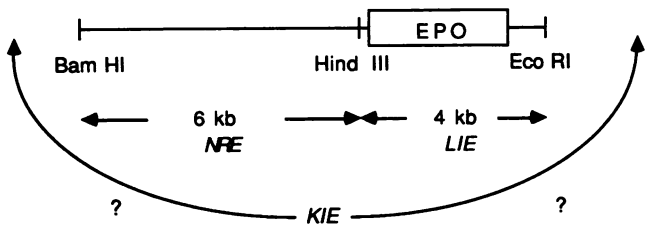

FIG. 8. Proposed location of human EPO gene *cis*-acting transcriptional regulatory elements. KIE, Kidney inducibility element(s); LIE, liver inducibility element(s); NRE, negative regulatory element(s); ?, location unknown.

regulatory elements is an essential feature of several tissue-specific, developmentally regulated genes (6, 33). The EPO gene has the 5'-FS organization of a housekeeping gene (high G+C content, absence of well-conserved TATA or CAT box, and multiple transcription start sites), suggesting that the tissue specificity of constitutive expression is dependent on negative regulation, whereas tissue-specific inducible expression is based on the presence of additional positive regulatory elements (which may also determine utilization of the downstream transcription initiation site). The proposed location of the three regulatory elements discussed above is summarized in Fig. 8.

Multiple human EPO RNA transcription initiation sites are utilized differentially. An unexpected result of our analysis of human EPO gene expression was the detection of multiple RNA transcription initiation sites. The downstream site maps to a sequence homologous to the mouse EPO gene start site, and its utilization generates a first exon of 250 to 252 nt which, in the mouse EPO gene, starts at either of two alternate cytosine residues (31), collectively referred to here as the downstream site. RNase protection analysis revealed multiple fragments protected by liver RNA from transgenic mice, all of which were more prominent in samples from anemic than nonanemic mice. The nucleotide sequence 5' to the upstream start sites shares homology with a sequence 5' to the downstream start site in the human and mouse EPO genes (Fig. 9). In the mouse EPO gene, a sequence with TATA-box homology, GATAACA, is located 22 to 24 nt 5' to cytosine residues at which transcription is initiated. At the same location in the human gene, this sequence is 22 to 24 nt 5' to cytosine residues which may serve as the downstream start site. At 232 nt further 5' to this sequence in the human gene is another TATA-box homolog present within 15 nt, 13 of which are identical to the downstream sequence. This upstream sequence is also 22 to 24 nt 5' to cytosine residues. The 72-nt protected fragment of pHP215 maps an upstream start site to the second of these cytosine residues. There are

MOUSE:	CTGATAACATCCCCG -15 nt- **C A C**
HUMAN (3'):	CAGATAACAGCCCCG -15 nt- **C G C**
HUMAN (5'):	CAGATAGCAGCTCCG -15 nt- **C G C**

FIG. 9. Analysis of nucleotide sequence 5' to EPO gene transcription initiation sites. Mouse, Sequence preceding the mouse EPO RNA initiation site mapped by S1 protection assay (31) to alternate cytosine residues (bold); human (3'), sequence at the corresponding location in the human EPO gene; human (5'), sequence located 232 nt upstream of human (3'); 15 nt, sequence of 15 nt conserved between mouse and human (3') only.

no additional TATA-box homologs further 5' to account for the larger protected fragments, suggesting either that the upstream TATA-box homolog is not strictly utilized or that nuclease digestion was incomplete, although increasing the RNase T_1 concentration two- and fivefold still resulted in multiple protected fragments (not shown). The same fragments are protected by RNA from hypoxic human hepatoma cells, indicating that the utilization of start sites is not solely a phenomenon of expression in transgenic mice but is also a property of the endogenous human EPO gene.

Liver RNA from tgEPO4 mice also protected full-length probe, consistent with the high-molecular-weight RNA identified by Northern blot analysis in tgEPO4 but not tgEPO10 mice. Protection of pHP215 to its 5' end (corresponding to the 5' end of the microinjected fragment) implies that the high-molecular-weight RNA is initiated within adjacent mouse DNA sequences. The RNA is inducible despite aberrant initiation, suggesting that mechanisms of initiation and induction are distinct. An additional 6 kb of 5'-FS in tgEPO10 mice allows for proper upstream initiation as well as restricting expression to the liver.

Transcription from the upstream and downstream human EPO gene start sites is regulated differentially, both in transgenic mice and in human cells expressing the EPO gene, suggesting that different promoters regulate transcription from the two sites, as is the case for several eucaryotic genes (27). The downstream start site is utilized in the liver of anemic transgenic mice and in Hep3B cells in which EPO gene expression has been induced by hypoxia. Base-line, noninduced transcription in transgenic mouse liver and human fetal liver, transcription in other transgenic tissues in which EPO gene transcription does not normally occur such as brain, and transcription in transgenic kidney, in which induction is not observed, all occur only from the upstream site. After induction, there is a marked increase in transcription from the upstream and downstream start sites both in transgenic mouse liver and in human hepatoma cells.

The function of multiple species of human EPO mRNA synthesized in anemic liver is unknown. Additional 5' untranslated sequences may affect translation efficiency, RNA stability, or RNA processing. RNA initiated from the upstream sites contains a 5' open reading frame, although the two published sequences (16, 21) differ, affecting its potential size. The transcription start site of the mouse EPO gene was originally assigned (22) 230 nt 5' to the site identified by S1 protection analysis (31) on the basis of homology to a transcription start site consensus sequence (7), the presence of a CAAT box, and two imperfect 11-nt repeats (22). It is possible that the mouse gene is initiated at several sites and that larger protected fragments were not detectable by the S1 nuclease assay as it was performed; alternatively, only the downstream initiation site may be utilized in anemic kidney, the source of RNA for the S1 nuclease analysis (31). It will be of interest to determine human EPO mRNA initiation site utilization in the kidney once regulated expression of the human EPO gene in transgenic kidney is achieved.

The transgenic mice reported here have provided insight into the nature of the *cis*-acting regulatory elements controlling human EPO gene expression. We are generating transgenic mice with other EPO gene constructs in order to further localize these regulatory elements, more clearly elucidate potential interactions of promoter, enhancer, and silencer elements with *trans*-acting factors in different tissues, and thus better understand the molecular basis for developmental-stage, tissue-specific regulated gene expression.

ACKNOWLEDGMENTS

We thank C. Shoemaker and Genetics Institute Inc. for providing human and mouse EPO genomic and cDNA clones, B. O'Hara of The Johns Hopkins University for the cloned mouse somatostatin gene, E. Fuchs of the University of Chicago for the human β-actin cDNA clone, M. Goldberg of Harvard University for Hep3B cells, and D. Driscoll of The Johns Hopkins University for human fetal liver. We are grateful to K. Hill and B Klaunberg for technical assistance and to H. H. Kazazian, M. B. Penno, and A. F. Scott for manuscript review.

This work was supported in part by Public Health Service grants HL-07983-01 (to G.L.S.) and DK-39869-02 (to S.E.A.) from the National Institutes of Health. G.L.S. is a Lucille P. Markey Scholar, and this work was supported in part by a grant from the Lucille P. Markey Charitable Trust.

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