

# Erythropoietin Messenger RNA Levels in Developing Mice and Transfer of <sup>125</sup>I-Erythropoietin by the Placenta

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## Abstract

Erythropoietin (EP) mRNA was measured in normal and anemic mice during fetal and postnatal development. Normal fetal livers at 14 d of gestation contained a low level of EP mRNA. By day 19 of gestation, no EP mRNA was detected in normal or anemic fetal livers or normal fetal kidneys, but anemic fetal kidneys had low levels of EP mRNA. Newborn through adult stage mice responded to anemia by accumulating renal and hepatic EP mRNA. However, total liver EP mRNA was considerably less than that of the kidneys. Juvenile animals, 1–4 wk old, were hyperresponsive to anemia in that they produced more EP mRNA than adults. Moreover, nonanemic juveniles had readily measured renal EP mRNA, whereas the adult level was at the lower limit of detection. Because of the very low level of fetal EP mRNA, placental transfer of EP was evaluated. When administered to the pregnant mouse, <sup>125</sup>I-EP was transferred in significant amounts to the fetuses. These results indicate that in mice the kidney is the main organ of EP production at all stages of postnatal development and that adult kidney may also play some role in providing EP for fetal erythropoiesis via placental transfer of maternal hormone.

## Introduction

Erythropoietin (EP)<sup>1</sup> is a glycoprotein hormone that regulates the terminal stages of mammalian erythropoiesis. The source of EP in the body has been previously sought by measuring the hormone in various tissues using either bioassays or radioimmunoassays. Although these studies may have indicated where EP is located or stored in the body, the identification of the tissues that produce EP requires other experimental approaches. The recent clonings of the murine EP gene (1, 2) have provided probes with which to identify the organs involved in EP production by measuring their content of mRNA coding for EP. We have found in anemic adult mice that ~85–90% of EP mRNA is in the kidneys and 10–15% is in the liver (3). Others have found a more equal EP mRNA distribution between liver and kidneys in cobalt-treated rats (4).

An important issue that is not yet understood is the source of EP during prenatal and early postnatal development. The production of EP during fetal and postnatal development has been examined mainly in sheep and rats. EP assays in

nephrectomized and/or hepatectomized sheep (5, 6) suggested that the livers of the anemic fetuses accounted for almost all of the EP until late in gestation when the kidneys accounted for a small portion of the total fetal EP. Through birth and the neonatal period the kidneys accounted for an ever-increasing portion of the total EP in anemic sheep. Similar types of experiments using hypoxic, neonatal rats, found that the kidneys appeared to account for little of the EP that could be bioassayed in the plasma in the first few days of life (7–9). After the neonatal period, however, the kidneys produced progressively greater amounts of EP during the first four weeks of life. In hypoxic rats three weeks old or less, Gruber et al. (7) suggested that the liver was responsible for some production of EP. However, no EP was detected by Fried et al. (8) or Caro et al. (9) in bioassays of EP in the livers of hypoxic newborn and developing rats. A more recent study using radioimmunoassays (10) in hypoxic fetal and neonatal rats suggested that the major portion of EP is produced by some tissue other than kidneys or liver in mid-gestation, by the liver in later gestation, and by the kidneys just before birth and during postnatal development. Any transplacental contribution of maternally produced EP to the fetus in these sheep and rat studies could not be determined. Although some studies examining the possible fetal responses to the transfer of EP from the mother did not support such a transfer (11, 12), the direct measurement of fetal mouse EP activity after administration of large doses of EP to the mother did indicate that some placental transfer does occur (13). The recent availability of large amounts of pure recombinant EP, which can be radiolabeled, provides a new tool to study placental transfer of EP.

We report here the analysis of EP mRNAs to determine which tissues produce EP in normal and anemic mice during fetal and postnatal development. Using <sup>125</sup>I-labeled EP, we also examine the placental transfer of EP from mother to fetus.

## Methods

*Mice.* Normal and timed pregnant BALB/c mice were purchased from Cumberland View Farms, Clinton, TN. Anemia was induced in mice either by removal of 0.5 ml of blood under ether anesthesia or by intraperitoneal injection of from 40 to 75 µg of phenylhydrazine hydrochloride per gram of body weight. Bled mice were volume repleted with 0.5 ml of isotonic saline. These treatments were done at 36, 24, and 12 h before sacrifice. Hematocrits were determined just prior to sacrifice, and those mice with hematocrits of 16–22% were used as anemic mice. Normal controls had hematocrits ranging from 46 to 50% for adults (60 d old), 33–37% for newborns, 32–35% in 9-d-olds, 29–33% in 16-d-olds, and 38–42% in 30-d-olds. For fetal studies, the gestational ages were determined by vaginal plugging with the morning of the plug designated as day 1 and the day of birth as day 20. Fetal anemia was induced by maternal injection of phenylhydrazine as in the above schedule. Due to fetal loss when the maternal hematocrits were reduced below 24%, the phenylhydrazine dosage was reduced to achieve 25–28% hematocrits. Fetal hematocrits on day 18 or 19 of

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1. Abbreviations used in this paper: EP, erythropoietin.

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gestation were also reduced to 25–28% with this treatment while normal fetal hematocrits were 34–38%.

**Analyses of EP mRNA.** For tissue RNA isolation, kidneys or livers were pooled separately from groups of animals of the following numbers: (a) animals 2 wk old or older, groups of 6 to 12; (b) animals 1 wk old, groups of 10 to 24; (c) newborn animals, groups of 20 to 35; (d) fetuses, groups of 40 to 77. Total RNAs from the mouse organs were obtained by homogenization in 4 M guanidine thiocyanate and sedimentation through 5.7 M CsCl as in the method of Chirgwin et al. (11). Polyadenylated RNAs were isolated from total RNAs by chromatographic separation with oligo (dT)-cellulose. From 1 to 6 mg of total RNA was isolated from each pool of organs and for isolation of polyadenylated RNAs, typically 1 mg of total RNA was loaded onto a column (1 cm diam by 3 cm length) of type 3-oligo (dT)-cellulose (Collaborative Research, Lexington, MA). The quantities of RNAs were determined by absorbance measurements at 260 nm, and the samples were aliquoted and stored as alcohol precipitates in 1.5 ml microcentrifuge tubes pending analysis. The RNAs were separated by electrophoresis on formaldehyde-1.5% agarose gels (15) and blotted onto nitrocellulose sheets.

The blotted RNAs were hybridized (16) to a DNA probe of the EP gene that had been radiolabeled with [ $\alpha^{32}$ P]dCTP by nick translation (17) to a specific activity of  $10^8$  cpm/ $\mu$ g DNA. The DNA probe was the 2,783 bp Bam HI-Eco RI restriction fragment of a 4.5-kbp genomic clone of murine EP (2, 3). Autoradiographs of the hybridized blots were scanned with a laser densitometer to determine the relative amounts of hybridizing EP mRNA in each sample. Results were expressed relative to a standard RNA preparation from anemic adult kidneys for which the EP mRNA was assigned a value of 1.0. This standard RNA preparation was used throughout the studies as a positive control.

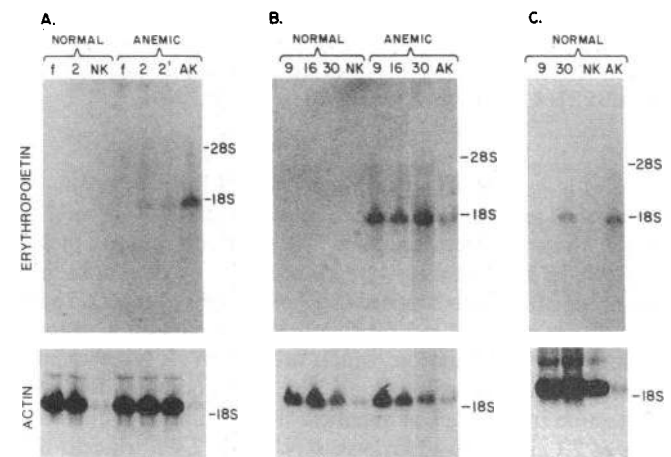
Several steps were taken to ensure that the stated amounts of RNA were actually loaded onto each gel lane and that this RNA was intact and transferred uniformly to the nitrocellulose blots. Firstly, the equivalence of total cellular RNA samples to be loaded onto a gel was confirmed by running "mini" formaldehyde-agarose gels of 5- $\mu$ g portions of each sample and staining the gels with ethidium bromide to compare the 28 S and 18 S ribosomal RNAs. And after analysis of the Northern blot with the EP specific probe, this probe was removed by immersing the blot in 0.015 M NaCl, 0.0015 M sodium citrate for 10 min at 85°C, and the same blot was rehybridized with a nick-translated DNA probe specific for cytoplasmic actin (18). Finally after a second round of probe removal, the blots containing total cellular RNAs were hybridized with a probe specific for 28 S ribosomal RNA (18). Hybridization of the total cell RNA blots with the ribosomal RNA probe (not shown) revealed that similar amounts of total RNA were present on all lanes of these blots, and these controls coupled with the hybridization of the actin probe on all blots, including those containing polyadenylated RNAs, showed that all lanes contained undegraded RNAs in the appropriate amounts.

Messenger RNA for EP was also detected by RNase protection assays. A 2,125-bp Bam HI-Sma I gene fragment contained within the Bam HI-Eco RI fragment described above was inserted into the poly-linker sequence of pGEM-1 (Promega Biotec, Madison, WI). After cleavage of the recombinant plasmid with Bam HI to linearize the DNA (leaving the EP-related fragment at one end), T7 bacteriophage RNA polymerase was used to transcribe a labeled RNA probe complementary to the EP mRNA in a modification of the method of Melton et al. (19). Labeling was accomplished using [ $\alpha^{32}$ P]UTP (800 Ci/mmol) to yield a RNA probe with a specific activity of about  $1 \times 10^9$  cpm/ $\mu$ g. The labeled RNA probe was hybridized with 50  $\mu$ g of total cellular RNA or 5  $\mu$ g of polyadenylated RNA in 25  $\mu$ l of 40 mM Pipes, pH 6.7, 0.4 M NaCl, 1 mM EDTA, 80% formamide at 40°C for 18 h. The hybridized RNAs were digested with RNases A and T<sub>1</sub> and analyzed on a denaturing 8% acrylamide-8 M urea gel. Conditions for RNase digestion were 40  $\mu$ g/ml of RNase A and 20 U/ml of RNase T<sub>1</sub> in a reaction volume of 300  $\mu$ l at 37°C for 1 h.

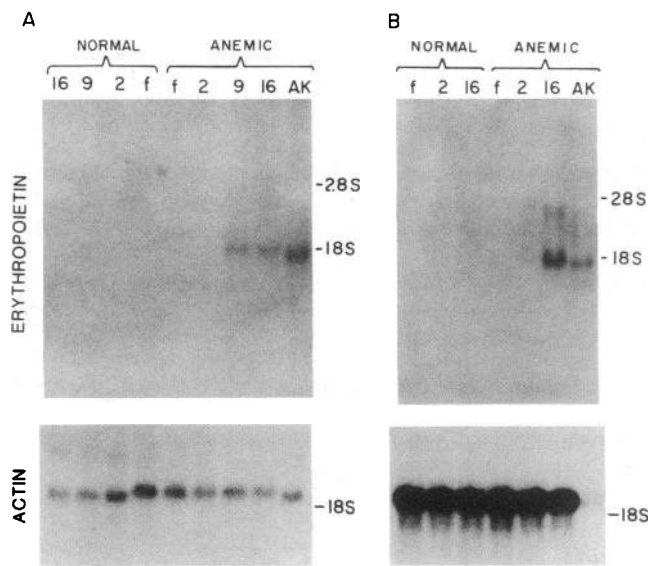
**Analysis of maternal-fetal transfer of  $^{125}$ I-EP.** To measure maternal-fetal transfer of EP, day 19 pregnant mice were given  $5 \times 10^6$  cpm (1.125 U) of  $^{125}$ I-EP via tail vein injection. The EP was pure recombinant human EP (129,000 U/mg; AMGen Biologicals, Thousand Oaks, CA) which was iodinated with Na $^{125}$ I using Iodo-Gen (Pierce Chemicals, Rockford, IL) as previously described (20). Over 95% of the biological activity of EP was retained after iodination. The pregnant mice were sacrificed at 1, 3, 6, and 10 h following the  $^{125}$ I-EP injection and their fetuses removed. Blood samples were obtained from the pregnant mice immediately before sacrifice. Each fetus was separated from its surrounding membranes, clamped at the umbilical cord, rinsed in three changes of fresh isotonic saline to remove any maternal blood, blotted dry, and fetal blood recovered by jugular vein laceration. The percentage of total  $^{125}$ I in the form of  $^{125}$ I-EP was determined by taking the sera from the blood samples and separating them on a TSK-3000 high pressure gel permeation column (Bio-Rad Labs, Richmond, CA). As a confirmation that all of the counts associated with high molecular weight material in the serum samples were in  $^{125}$ I-EP, aliquots of sera were separated by SDS-PAGE (21).

## Results

**Accumulation of EP mRNA in mouse organs during fetal and postnatal development.** Figs. 1 and 2 show autoradiographs of Northern blots using RNAs from kidneys and livers of mice at various prenatal and postnatal stages of development. As had been seen previously in adult mice (3), the major EP mRNA



**Figure 1.** Kidney EP mRNA in fetal and postnatal mice. Autoradiographs are shown for RNAs from murine kidneys that were electrophoretically separated, blotted onto nitrocellulose, and hybridized to a  $^{32}$ P-labeled DNA probe for EP as described in Methods. After removal of the EP probe as described in Methods the same blots were re probed for actin mRNA. Locations of 28S and 18S ribosomal RNAs are indicated. The blots were exposed with an intensifier screen for 7–10 d with the EP probe and for 10–48 h with the actin probe. Some mice were made anemic by bleeding or phenylhydrazine injection. Hematocrits of anemic mice were 16–22% except for fetuses and some 2-d-old newborns (lane 2) whose hematocrits were 25–28%. (A) Perinatal RNAs (50  $\mu$ g total RNA per lane): 19-d gestation fetuses (f), 2 d postnatal (2), 60-d-old adult control normal kidneys (NK) and anemic kidneys (AK). (B) Postnatal RNAs (50  $\mu$ g total RNA per lane): mice with postnatal ages of 9, 16, and 30 d as indicated, and adult controls (NK and AK). (C) Polyadenylated RNAs: 20  $\mu$ g polyadenylated RNA from normal kidneys at 9, 30, and 60 d postnatal; AK control is 20  $\mu$ g total RNA of 60-d-old anemic kidney.



**Figure 2.** Liver EP mRNA in fetal and postnatal mice. Autoradiographs of liver RNA blots hybridized with probes for EP and actin as described in Methods and Fig. 1 legend are shown. Some mice were made anemic as described in Fig. 1 legend. (A) Total liver RNAs (50  $\mu$ g total RNA per lane): 19-d gestation fetuses (f), mice with postnatal ages of 2, 9, and 16 d, and control adult, 60-d-old anemic kidney (AK). Small differences in migration of RNAs are due to slight differences in sample loading buffer rather than actual RNA size differences. (B) Polyadenylated liver RNAs: 19-d gestation fetuses (f), 2 days postnatal (2), and 16 d postnatal (16), and AK control from adult, 60-d anemic mouse. All lanes are loaded with 20  $\mu$ g polyadenylated RNA except AK control of 20  $\mu$ g of total RNA.

species is  $\sim 2$  kb in length. Kidneys and livers of postnatal mice of all ages contained measurable quantities of EP mRNA when the mice were anemic. The relative amounts of EP mRNA in the samples were determined by densitometric scanning of the 2-kb bands in autoradiographs (Table I). These figures are standardized relative to the signal seen in a total kidney RNA preparation, which was run in all experiments, from anemic 60-d-old animals with hematocrits of 18–22%. Detection in the livers of anemic 2-d-old animals required the use of 20  $\mu$ g of polyadenylated RNA (Fig. 2 B), whereas the mRNA levels from anemic tissues of other postnatal stages could be seen using total RNA. Using Northern blots, the only sample other than those from anemic postnatal animals which was positive for EP mRNA was the polyadenylated RNA from kidneys of 30-d-old normal animals (Fig. 1 C).

Actin probe hybridization on blots of Figs. 1 and 2 serves as a control for insuring that intact RNA was transferred on the lanes. Actin mRNA levels in the kidneys are high in newborns and very young animals, and the levels drop as the animal's age increases, reaching a much lower level in adult mice (Fig. 1, A–C). Note that animals of comparable age, whether anemic or normal, have comparable levels of actin mRNA in the kidneys. Also, actin mRNA was present in the same relative proportions among samples on the blots containing total cellular RNAs as on those containing polyadenylated RNAs. The levels of actin mRNA in livers of developing animals also show progressive decrease during the first few weeks after birth although the changes in the liver are not as drastic as those seen

in the kidneys (Fig. 2, A and B). This developmental pattern for actin mRNA in liver was reported earlier (18).

To further investigate EP mRNA levels in those tissues which showed no or weak signals on Northern blots, a more sensitive RNase protection assay was used (Fig. 3) with a labeled RNA probe that included exons 2–4 of the EP gene. The three exons, which are protected from RNase in Fig. 3, are indicated at lengths of 180, 143, and 87 bases. A fourth band on the gel is marked by an asterisk at a length of 110 bases. The relative molar decrease in the 143-base band as compared to the 180 and 87 base ones suggest that the asterisk-marked band represents a shortened [by about 30 bases] protected species of the 143 base band due to either incomplete transcription in the formation of the labeled probe or some sequence structure that allows RNase digestion at this point. Using the 180 base bands for comparison, densitometric scanning yielded amounts of EP mRNA in the test samples relative to that in the control "AK" sample from anemic adult kidney (i.e., same control as for Northern blots). Several tissue RNA preparations have been compared to that from adult anemic kidney by both

**Table I.** Relative Amounts of EP mRNA in Kidneys and Livers of Mice

Age	EP mRNA/ $\mu$ g total RNA*		Relative EP mRNA <sup>†</sup>	
	Kidney	Liver	Kidney	Liver
<i>d</i>				
Anemic postnatal <sup>‡</sup>				
2	0.245	0.006	11	3
9	2.05	0.125	232	88
16	2.41	0.129	319	100
30	5.26	0.100	863	89
60	1.00	0.015	212	16
Normal postnatal				
2	Und <sup>  </sup>	Und		
9	0.004	Und	0.5	
16	0.007	Und	0.9	
30	0.037	Und	6.1	
60	0.002	Und	0.4	
Fetal <sup>††</sup>				
14		0.004		0.1
16		Und		
19	Und	Und		
(19 d anemic)	0.011	Und	0.2	

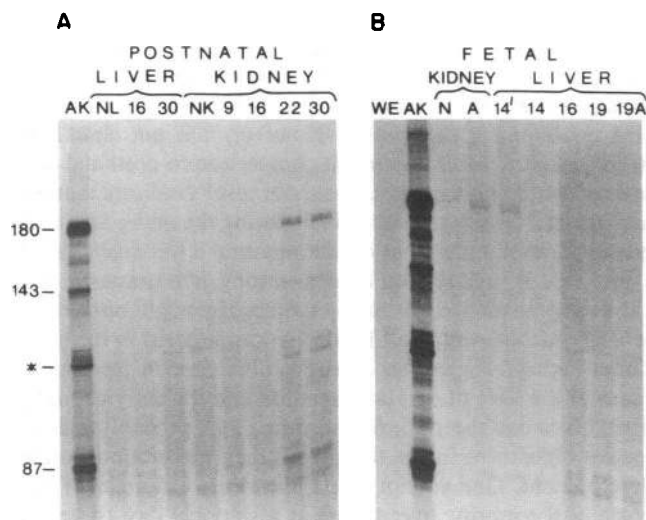
\* EP mRNA values are expressed relative to anemic adult (60 d) kidney which is assigned a value of 1.00.

<sup>†</sup> Relative EP mRNA is the product of EP mRNA/ $\mu$ g total RNA and total  $\mu$ g of RNA per 2 kidneys or 1 liver. The average quantities of total RNA in  $\mu$ g per 2 kidneys or 1 liver in postnatal mice were respectively: 2 d, 42 and 424; 9 d, 113 and 704; 16 d, 133 and 758; 30 d, 164 and 885; 60 d, 212 and 1,071. Average for 2 kidneys in day 19 gestation fetal mice was 22  $\mu$ g and fetal liver at day 14 of gestation was 30  $\mu$ g.

<sup>‡</sup> Anemic postnatal mice have hematocrits of 16–22%.

<sup>||</sup> Und, undetectable by methods used (< 0.002 of adult anemic kidney).

<sup>††</sup> Ages for fetuses are days of gestation; fetal kidneys cannot be identified prior to day 17; anemic fetuses have hematocrits of 25–28%.



**Figure 3.** Autoradiograph of EP mRNA protection assay. 50- $\mu$ g aliquots of total cellular RNAs from various fetal and postnatal mice were hybridized to a complementary  $^{32}$ P-labeled ribonucleotide EP probe, RNase-digested, and separated on a urea-acrylamide gel as in Methods. The EP probe was protected at three areas corresponding to exons of 180, 143, and 87 bases. Asterisk indicates band that appears to be derived from the 143-base exon as described in Results. (A) Postnatal tissues. Lanes are labeled with the day of postnatal life when the samples were obtained from normal (nonanemic) kidneys and livers. NL and NK are the liver and kidney RNA from normal 60-d-old adults. AK is RNA from 60-d adult anemic kidney. (B) Fetal tissues. Kidney RNAs are from day 19 gestation mice and are labeled (N) from normal fetuses and (A) from anemic fetuses. Liver RNAs are from normal fetuses at days 14 through 19 of gestation except for lane 19A, which is from an anemic day 19 fetus. Lane 14' in B is 5  $\mu$ g of polyadenylated RNA. WE is whole embryo minus the liver from day 14 of gestation and AK is 60-d adult anemic kidney.

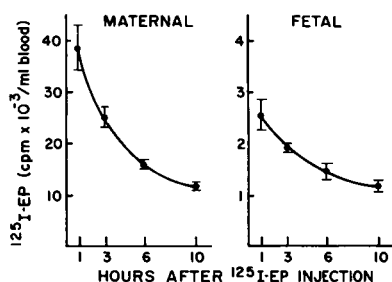
Northern blotting and RNase protection assays and the relative EP mRNA levels have been quite similar by both methods, indicating that both yield autoradiographic signals that are directly proportional to mRNA concentration under the conditions used.

The data presented above used RNA preparations obtained from large groups of animals to minimize the effects of individual variation and to avoid working with trace quantities of RNA. Each of the experiments has been repeated with RNAs isolated from at least three additional equivalent groups of animals. Figs. 1–3 and Table I can be summarized in relation to the developmental sequence of fetal through adult stages. EP mRNA was detectable in normal fetal livers at day 14 of gestation, but was never found in fetal livers on days 16 and 19 of gestation (Fig. 3 B). Whole fetuses minus the fetal livers at day 14 of gestation were negative for EP mRNA (lane WE in Fig. 3 B) as were the placentas (data not shown) from these fetuses. At day 19 of gestation, the fetal kidney responded to anemia by accumulating EP mRNA, while no EP mRNA was detected in the anemic fetal liver (Fig. 3 B). These results suggest that the kidney has become the most responsive organ to anemia in the fetus at one day after it can be identified macroscopically. 2-d postnatal, anemic mice have both renal and hepatic EP mRNA (Figs. 1 and 2). In Table I, the amount of EP mRNA per microgram of total RNA in the 2-d-old

anemic mouse kidney is one fourth, and in the liver one half, of that found in the respective organs of an anemic, young adult (60-d-old) mouse with a similar hematocrit. At 9 d of age, the anemic mouse raises its renal EP mRNA per microgram RNA to twice that found in the anemic adult kidney and its hepatic EP mRNA to eight times that found in the anemic adult liver (Table I). This relatively increased response to anemia by accumulation of EP mRNA per microgram RNA continues through 16 d of age and reaches values in the kidneys and livers of anemic 30-d-old mice which are five times more than the respective anemic adult organs. It is only around this 30-d postnatal period that EP mRNA could be detected by Northern blotting in the kidneys of normal mice (Fig. 1 C) although by the RNase protection assay EP mRNA was detected in normal animals at 9, 16, and 22 d of age as well (Fig. 3 A). The EP mRNA level in nonanemic adult (60-d-old) kidneys is at the lower limit of detection by RNase protection. An extremely faint band at the 180-base exon position can be seen in the NK lane of Fig. 3 when exposure time is increased fourfold over the exposure shown. By densitometry of variable duration exposures this NK signal is about 0.002 of the signal from anemic 60-d-old mice (Table I). Therefore, an  $\sim$  500-fold increase in EP mRNA appears to occur with the induced anemia in adult mice. A much more accurate comparison between the normal and anemic levels of kidney EP mRNA can be made in the case of the 30-d postnatal mouse where the anemic mouse has a value 5.26 and the normal has a value of 0.037 (see Table I). Thus, a greater than 140-fold increase in renal EP mRNA occurs when the hematocrit is lowered to the 16–22% range in these mice.

From the relative amounts of EP mRNA per microgram total RNA and the total RNAs recovered from livers and kidneys at the various ages, the distribution of total EP mRNA between the kidneys and liver can be assessed during murine development (Table I). The total cellular RNA in newborn liver is approximately 10 times as much as in both kidneys. However, the kidneys develop rapidly, and by 30 d of postnatal life the adult ratio of about five times as much hepatic as renal total RNA is achieved (Table I footnote). In anemic mice, the kidneys account for about 75% of the total EP mRNA in the first 2 wk of postnatal life, while by 30 d of age and later they account for about 90% of the EP mRNA detected (Table I).

*Transfer of maternal EP to the fetus.* The possible transfer of EP from the mother to the fetus via the placenta was investigated by giving day 19 pregnant mice a single intravenous injection of  $5 \times 10^5$  cpm of  $^{125}$ I-EP. This amount represented a total of 1.125 biological units of EP per mouse. At various times after the injections, the pregnant mice were sacrificed and maternal and fetal blood samples taken. Aliquots of sera from the blood samples were separated on a TSK-3000 column to determine the proportion of total counts per minute in iodinated EP as opposed to iodotyrosine or iodide. When the sera were separated on SDS-polyacrylamide gels, a single 35,000 molecular weight band migrating identically to  $^{125}$ I-EP was recovered. The  $^{125}$ I-EP crossed the murine placenta and achieved concentrations in the fetal blood of from 7 to 10% of that found in the maternal blood at the same time (Fig. 4). The  $^{125}$ I-EP had similar disappearance rates in the maternal and fetal blood. During the 10 h after the injection of the pregnant mice, the half-life of  $^{125}$ I-EP is  $\sim$  6 h in both maternal and fetal circulations (Fig. 4).



**Figure 4.** Maternal-fetal transfer of  $^{125}\text{I}$ -EP. Day 19 pregnant mice were injected with  $5 \times 10^5$  cpm of  $^{125}\text{I}$ -EP (1.1 biological unit) at 0 h. At 1, 3, 6, and 10 h after the injection, three mice were sacrificed and fetal and maternal blood collected as in

Methods. Fetal blood from all members of a litter were pooled. The  $^{125}\text{I}$ -EP in the blood samples was determined as in Methods. Data are shown as means  $\pm$  1 SD.

## Discussion

The results demonstrate that the kidneys, as opposed to the liver, account for the majority of EP mRNA found in anemic mice from the newborn through adult stages of murine development. During the first month of postnatal life, the proportion of total EP mRNA accounted for by the kidneys increases from about 75 to 90% (Table I). Much of this increase in the renal proportion of total EP mRNA is probably due to the rapid morphological (22) and functional (23) development of the kidney during the first few weeks of rodent postnatal life. In addition to this increasing proportion of kidney EP mRNA during postnatal development, an altered responsiveness to anemia depending upon age is apparent in the EP mRNA of both the kidneys and the livers of developing mice (Table I). Neonates respond to anemia by accumulation of relatively small levels of EP mRNA. However, the relative hyporesponsiveness to anemia in neonates appears to be transient. Indeed, the EP mRNA per  $\mu\text{g}$  total RNA in the kidneys and livers of 9-day through 30-day postnatal mice actually demonstrate a hyperresponsiveness to anemia when compared to adults with similar hematocrits of 16 to 22% (Table I). In anemic rats, Caro et al. (9) found that renal EP levels followed a similar hyperproduction pattern during the first month of life. This period of increased responsiveness to anemia is one of very rapid growth as all of the mice increase their birth weight by greater than 10-fold. In this regard, in hypoxic, nephrectomized rats, regenerating liver has been reported to produce much more EP than intact normal liver (24). Thus, those tissues that produce EP appear to have an increased responsiveness to anemia when they are undergoing active growth or regeneration. It is significant that during this period of hyperresponsiveness to anemia the renal EP mRNA levels of non-anemic animals are much higher than in adult animals (Figs. 1 C and 3). At this point in development (1–4 wk postnatal), the mice reach their physiological nadir in hematocrits, with a normal average of 30% at 22 d of postnatal age compared to the normal adult value of 48%. Thus, although EP mRNA is detected in the kidneys of these normal developing mice, they may be considered to have a "physiological anemia."

In accordance with the work of others, the fetal liver appears to be one source of EP during gestation. Previous studies by others in fetal sheep (5, 6) and rats (10) suggested the liver as a source. EP mRNA has been demonstrated in the liver of a human fetus of 20 wk gestation (25). This time point is in the middle trimester of human gestation, corresponding to approximately day 10 or 11 of murine gestation. In the present study, EP mRNA was detectable in normal murine fetal livers

at gestational day 14. Despite testing multiple groups of fetuses, no EP mRNA was detectable in fetal livers on days 16 through 19 of gestation, even when the fetuses were rendered moderately anemic (hematocrits of 25–28%) by phenylhydrazine treatment of the mother. However, it is not clear how hypoxic these fetuses become in comparison to postnatal animals of similar hematocrits. Thus, our results indicate that the murine fetal liver is a source of EP during the mid-gestational period of development but by late gestation it has much less of a role in EP production. Indeed, during late gestation, the kidneys responded to anemia by synthesizing slight but detectable EP mRNA while no EP mRNA was detected in the liver. These findings suggest an apparent difference in the significance of the liver during late gestation and the early neonatal period between sheep (5, 6) and mice. This probably reflects species differences in the timing of organ maturation during development. One cannot assess the relative degrees of hypoxia of the murine liver tissues at gestational day 14 versus gestational day 19, so it is not possible to distinguish whether it is the stimulus or the capacity of the liver to respond which leads to the observed differences in EP mRNA accumulated. Although the EP mRNA in the gestational day 14 liver is relatively low (Table I), the liver is the major site of erythropoiesis at this stage of fetal development. Thus, the local levels of EP in the gestational day 14 liver may be quite elevated as compared to other tissues. The results of this work do demonstrate that the accumulation of EP mRNA in response to the same degree of anemia is increased dramatically as the developing mouse progresses from a fetus 1 d before birth to a newborn 2 d after birth (compare lanes *f* and *z* in Fig. 1 A). Furthermore, this responsiveness continues to increase dramatically during the first 4 wk of life, and later decreases somewhat to the adult state.

The results in Fig. 4 shows that some EP is transferred from the mother to the fetuses. At all times tested after maternal injection of  $^{125}\text{I}$ -labeled EP (1–10 h), the  $^{125}\text{I}$ -EP concentration in fetal blood was about 7–10% of the level in maternal blood. However, in both mice and humans, the fetal erythroid progenitor cells have been shown to be more sensitive to EP than those of adults (26, 27) and, therefore, this lower level of EP in fetal blood may be sufficient for a normal fetal erythropoietic response. The amount of  $^{125}\text{I}$ -EP administered to the pregnant mice resulted in EP blood levels which were higher than normal, an estimated 250 mU/ml immediately after injection and a measured 90 mU/ml at 1 h postinjection. However, these values are within the range achieved in mild to moderate anemia and are much less than those seen in severe anemia (9, 10). Since iodination of EP to higher specific activities than that used here destroys binding and biological activity (20), placental transfer of EP at basal physiological levels cannot be evaluated in mice. However, the placental transfer of EP in conditions similar to mild anemia suggest that it may have a physiological role in fetal erythropoiesis. Preliminary work in our laboratory indicates that  $^{125}\text{I}$ -EP binds specifically to receptors on the murine placenta (unpublished results). Although these preliminary results suggest that placental receptors for EP may be involved in transfer of EP from the mother to fetus during late gestation, further studies will be required to prove this transfer function.

The data presented here show that in the mouse (a) the fetal liver produces low levels of EP mRNA during mid-gestation that decline during late gestation, (b) by the last day of

gestation, the fetal kidney is responsive to anemia by EP mRNA production, (c) the kidney is the main organ of EP production at all stages of postnatal development, and (d) the maternal kidney may play a role in providing some proportion of the EP for fetal erythropoiesis via placental transfer of maternal hormone.

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