Anemia Induces Accumulation of Erythropoietin mRNA in the Kidney and Liver

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Regulation of the production of erythropoietin occurs in the kidney and liver largely through control of accumulation of erythropoietin mRNA. Erythropoietin mRNA was first detected in kidneys at 1.5 h postanemia and reached a plateau value at least 200-fold above the control value by 4 to 8 h. A 20-base sequence immediately upstream from the reported erythropoietin mRNA initiation site is complementary to a hypervariable sequence in 18S rRNA.

Erythropoietin (EP) is a glycoprotein hormone which is the principal regulator of mammalian erythrocyte development. Studies measuring the biological activity of EP have demonstrated that the kidney plays a predominant role in raising circulating EP titers in response to hypoxia (6, 7). In hypoxic animals, EP can be extracted from renal tissues but not other tissues (2, 3). The only other organ which has been implicated in EP production is the liver of fetal (14) or nephrectomized (1, 3, 8) animals. The recent cloning of the mouse EP gene (12) provides the probes necessary for analysis of the organ distribution, quantitation, and time course of accumulation of EP-specific mRNA transcripts in mice made hypoxic by blood loss.

RNA was isolated from mouse organs by homogenizing them in guanidine isothiocyanate and sedimenting the RNA through 5.7 M CsCl (4). Polyadenylated RNA was isolated by chromatography with oligo(dT)-cellulose. For electrophoresis, 6 to 25 µg of polyadenylated RNA or 30 to 50 µg of total cellular RNA was loaded per lane on formaldehyde-1.5% agarose gels (9). After electrophoresis, the gels were blotted onto nitrocellulose sheets and hybridized by the method of Thomas (13), except that no dextran sulfate was added to the hybridization buffer. DNA probes were labeled by nick translation (11) to a specific activity of 10^8 cpm/µg of DNA.

Figure 1 depicts the structure of the mouse EP gene. Plasmid DB2-5, which has the indicated 4.5-kilobase-pair *Bgl*II fragment of the mouse EP gene inserted into the *Bam*HI site of the plasmid vector pUC19 (12), was a gift from C. Shoemaker, Genetics Institute, Cambridge, Mass. The inserted *Bgl*II fragment contains the entire promoter and coding regions of the gene (and most of the 3' untranslated sequence). The probe used to detect EP-specific transcripts was the *Bam*HI-*Eco*RI fragment or the *Bam*HI-*Sma*I fragment prepared from DB2-5 by digestion and isolation from a 1% agarose gel (Fig. 1). The former probe is more sensitive than the latter, but in addition to EP mRNA it also hybridizes with a small nucleic acid species, present in equal amounts in all mouse cells, which migrates diffusely with the dye front in formaldehyde-agarose gels.

For studies of the organ distribution of EP mRNA, female BALB/c mice aged 12 to 14 weeks and weighing 20 to 24 g were made anemic by bleeding from the retroorbital sinus. The mice were bled 0.4 ml at 36, 24, and 16 h before

sacrificed by cervical dislocation. Their hematocrits just before sacrifice were 17 to 20%, whereas hematocrits of normal animals were 48 to 51%. The results in Fig. 2 demonstrated that EP mRNA accumulation is strongly induced in the kidney by anemia. The main EP-specific species is about 2 kilobases in length, and a transcript of larger size (asterisk) is present in much smaller amounts. With high amounts of polyadenylated RNA (25 µg) and long exposure times, EP mRNA induction was also observed in the liver (Fig. 2, lanes 12 and 13). In both normal and anemic animals, no EP transcripts were observed in organs other than the kidney and liver. Other organs tested included spleen, brain, skeletal muscle, and lung. A 25-µg sample of polyadenylated RNA from normal kidney (Fig. 2, lane 10) contained a faint band at the position of EP mRNA, although the signal is at the limit of sensitivity and specificity of the Northern blot assay. Any EP mRNA in normal kidney must be at least 200-fold less than in anemic kidney.

Direct measurements of RNAs from the kidneys and livers of anemic mice showed that both kidneys contained a total of 192 µg of RNA, of which 2.4% was polyadenylated, and that the liver contained 970 µg, of which 4.6% was polyadenylated (average of three measurements). Densitometric comparisons of the signals in Fig. 2 showed that the anemic kidney polyadenylated RNA is about 60 times as rich in EP mRNA as anemic liver polyadenylated RNA. However, because the liver contains 10 times more polyadenylated

FIG. 1. Diagram of the mouse EP gene structure (12). Restriction endonuclease sites are as follows: B2, *Bgl*II; X, *Xba*I; B, *Bam*HI; S, *Sma*I; E, *Eco*RI; H, *Hind*III. Open boxes represent exons, and single lines depict flanking sequences and introns. The entire *Bgl*II gene fragment is approximately 4.5 kilobase pairs long. The EP probe fragment derived by cutting with *Bam*HI and *Sma*I is 2,125 base pairs, and that derived by *Bam*HI and *Eco*RI cutting is 2,783 bp. A short segment of the EP gene sequence is shown in which the boxed section represents a portion that is perfectly complementary to a sequence in mouse 18S rRNA.

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FIG. 2. Organ distribution of EP mRNA. Nitrocellulose blots (autoradiographs) of formaldehyde-agarose gels containing RNAs from various organs of normal (nonanemic) and anemic mice were hybridized with the *Bam*HI-*Sma*I probe (lanes 1 through 8) or the *Bam*HI-*Eco*RI probe (lanes 9 through 16) (Fig. 1). Each RNA preparation was from pooled organs of at least five mice. Lanes 1 through 4 contained, respectively, 30 µg of total RNAs from nonanemic liver, anemic liver, nonanemic kidney, and anemic kidney. Lanes 5 through 8 each contained, respectively, 6 µg of polyadenylated RNA from nonanemic liver, anemic liver, nonanemic kidney, and anemic kidney. The following lanes contain polyadenylated RNAs as indicated: 9, 2.5 µg of from anemic kidney; 10, 25 µg from nonanemic kidney; 12, 25 µg from nonanemic liver; 13, 25 µg from anemic liver; 14, 25 µg from anemic spleen; and 15, no RNA. Lanes 11 and 16 each contain 30 µg of total RNA from anemic kidney (same sample as lane 4), which served as a control standard in each blot. The origin is at the top of the figure. The locations of 18S and 28S rRNA are indicated. The asterisk indicates an additional, larger EP transcript.

RNA than both kidneys, the liver contains from 12 to 15% of the total EP mRNA in an anemic animal.

For studies of the time course of EP mRNA accumulation in the kidney, mice were bled by two different schedules. One group underwent a single 0.6-ml bleed followed immediately by repletion of blood volume by peritoneal injection of 0.6 ml of saline. This schedule reduced the hematocrits of the mice to 30 to 34% by 1 h after bleeding, and this hematocrit remained unchanged for the following 24-h period. Mice from a second group were bled 0.5 ml and immediately volume repleted with 0.5 ml of saline. After 30 min, they were again bled 0.5 ml and repleted with saline. This schedule reduced the hematocrits to 18 to 23% by 1 h after the second bleeding. Three mice from each group were sacrificed at various times after bleeding. No EP mRNA was detected at 1 h after bleeding in the animals with approximately 30% hematocrits, but it could be easily detected at 2 h and reached a maximum level by 4 to 8 h (Fig. 3A). Figure 3B shows results from animals bled to hematocrits of approximately 20%. A very faint band of EP mRNA is visible

FIG. 3. Time course of EP mRNA accumulation after blood loss. Shown are autoradiographs of nitrocellulose blots hybridized with the *Bam*HI-*Sma*I EP gene fragment (Fig. 1). (A) Autoradiograph of blotted gel containing in each lane 50 µg of total cellular RNAs extracted from kidneys of moderately anemic mice (hematocrits of 30 to 34%). The anemia was produced as described in the text, and the kidneys were removed at the times (in hours) postbleeding indicated above each lane. The lane marked C contained 30 µg of cellular RNA from kidneys of mice bled according to the schedule described for studying EP mRNA organ distribution (Fig. 1; hematocrits 17 to 20%). (B) Autoradiograph of blotted gel containing in each lane 30 µg of total cellular RNAs from kidneys of severely anemic mice (hematocrits of 18 to 23%). The times indicated above each lane are the times of kidney removal after the first of the two bleeds described in the text. The lane marked C contained 30 µg of the same RNA preparation as in lane C of panel A.

at 1.5 h, and accumulation is maximum by 4 to 8 h, as in the experiment of Fig. 3A. The lanes C in Fig. 3 represent quantitatively identical signals with which to compare the lanes of Fig. 3A with those of Fig. 3B. With a densitometer to determine the relative intensities of the signals in Fig. 3A and B, maximum EP mRNA levels in the kidneys of severely anemic mice (hematocrits about 20%) were found to be about 12-fold greater than levels in the moderately anemic mice (hematocrits about 30%). This result demonstrates a correlation between the severity of anemia and the level of EP mRNA accumulation.

When the *Xba*I-*Bam*HI fragment of plasmid DB2-5 (Fig. 1) was used to probe blots containing total mouse cell RNA, stable hybridization occurred with 18S rRNA. That the involved species was 18S rRNA was indicated by quantitatively similar hybridization with nonpolyadenylated RNA from all mouse tissues and mouse cell lines tested (data not shown). A comparison of the sequence of the *Xba*I-*Bam*HI EP DNA fragment (12) with 18S rRNA (10) revealed a 20-base sequence of perfect complementarity between the EP-coding DNA strand and 18S rRNA (Fig. 1). This sequence is immediately upstream from the reported EP mRNA cap site. The complementary sequence in 18S rRNA

is 257 to 276 bases from its 5' end in a region of 35 bases which constitutes a "hypervariable region" (10). If there were transcription start sites upstream from the one reported previously (12), this sequence could provide the basis of a strong interaction between the putative transcripts and ribosomes, thus promoting efficient translation of such an mRNA. That this sequence has some function in the regulation of EP gene expression is implied circumstantially by the extraordinary conservation of the upstream flanking sequences, including this specific sequence, in human and mouse EP genomic clones (5, 12).

These studies clearly demonstrate that in the adult mouse the kidney and, to a much lesser extent, the liver produce EP mRNA in response to anemia and its attendant tissue hypoxia. Since the level of EP mRNA in the kidney increases markedly within 2 h of blood loss and reaches a maximum level which correlates with the severity of anemia, it appears that the production of the hormone EP in the anemic adult mouse is regulated by the accumulation of its mRNA.

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