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Differential expression of *lacZ* in the liver and kidney of transgenic mice carrying chimeric *lacZ*–erythropoietin gene constructs with or without its 1.2 kb 3′-flanking sequence

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ABSTRACT

Erythropoietin (EPO) plays a key role in erythropoiesis and is expressed predominantly in the fetal liver and in the adult kidney. The *EPO* gene is up-regulated at the transcriptional level under hypoxic/anemic conditions. We studied the role of the 5′- and 3′-flanking sequences of the mouse *EPO* gene in its tissue-specific and hypoxia-induced expression by developing transgenic mouse lines carrying chimeric *EPO–lacZ* gene constructs. Transgenic mice carrying a 6.5 kb segment of the 5′-sequence and most of the *EPO* gene in which *lacZ* was substituted for exon 1 (5′-*lacZ–EPO*) demonstrated induction of *lacZ* expression following hypoxia/anemia induction in both the liver and kidney of adult mice. However, transgenic mice carrying the above construct along with the 1.2 kb 3′-flanking sequence (5′-*lacZ–EPO*-3′) showed a high level of *lacZ* expression following hypoxia/anemia induction in adult kidney but not in adult liver. With the aim of further understanding the role of the 3′-flanking sequence in tissue-specific expression of the *EPO* gene, we studied the interactions of protein factors with this 1.2 kb 3′ region and demonstrated that multiple sets of protein factors interact tissue specifically with a 10 bp sequence, TCAAAGATGG, located downstream of the previously characterized 3′ hypoxia-responsive enhancer element.

INTRODUCTION

Erythropoietin (EPO) is a glycoprotein hormone and the primary physiological mediator of erythropoiesis (1–3). It binds to specific cell surface receptors on erythroid progenitor cells and stimulates their proliferation, promotes differentiation and prevents apoptosis (4,5). In a recent report, EPO and EPO receptor (EPOR) knockout mice exhibited reduced primitive erythropoiesis and died around embryonic day 13, owing to failure of definitive fetal liver erythropoiesis. Both of these knockout mice exhibited identical phenotypes, indicating that EPO and the EPOR are crucial for definitive erythropoiesis *in vivo* and that no other ligand or receptors can replace them (6). In most species the liver is the major site of EPO synthesis during fetal life, whereas late in gestation the kidney becomes the predominant site of EPO production and remains so throughout life (2,7,8).

The enhancer sequences responsible for hypoxia induction of the *EPO* gene have been localized in both the 5′- and 3′-flanking sequence of the *EPO* gene (9–13). Hypoxia-inducible protein factors have been demonstrated to interact with these enhancer sequences (13–15). Hypoxia-inducible factor 1 (HIF1) was recently cloned and characterized as a basic helix–loop–helix–PAS heterodimer (16). The possible roles of hepatic nuclear factor 4 (HNF-4) and the COUP family of proteins in tissue-specific and hypoxia-inducible expression of the *EPO* gene have also been demonstrated (17). Maxwell *et al.* (12), using transgenic mice, showed that 9.0 kb 5′- and 3.5 kb 3′-flanking sequences contain the necessary regulatory elements for mouse *EPO* gene expression. Cooperative interaction between the 5′ promoter and the 3′ enhancer elements for hypoxia induction has been described before (14,18), but the 5′ and 3′ regulatory elements and their cooperative interactions in tissue-specific regulation of *EPO* gene expression are not well defined.

In this study we compared the pattern of expression of the *lacZ* reporter gene under normoxic and hypoxic conditions in the liver and kidney of transgenic mice carrying: the *lacZ* reporter linked to the 6.5 kb 5′-flanking sequence, the entire first exon and 200 bp of the 5′ portion of the first intron (5′-*lacZ*); the 6.5 kb 5′-flanking sequence and most (except exon 1 and 200 bp of intron 1) of the *EPO* gene (5′-*lacZ–EPO*); the 6.5 kb 5′-flanking sequence plus most of the *EPO* gene and the 1.2 kb 3′-flanking sequence (5′-*lacZ–EPO*-3′) of the mouse *EPO* gene. We provide evidence from these transgenic mouse lines that the 1.2 kb 3′-flanking region may contain a regulatory element for suppressing *EPO* gene induction in response to hypoxia/anemia in the adult liver. Also, evidence from our DNA–protein interaction studies show that sets of protein factors interact in a tissue- and development-specific pattern to a 10 bp sequence (TCAAAG-

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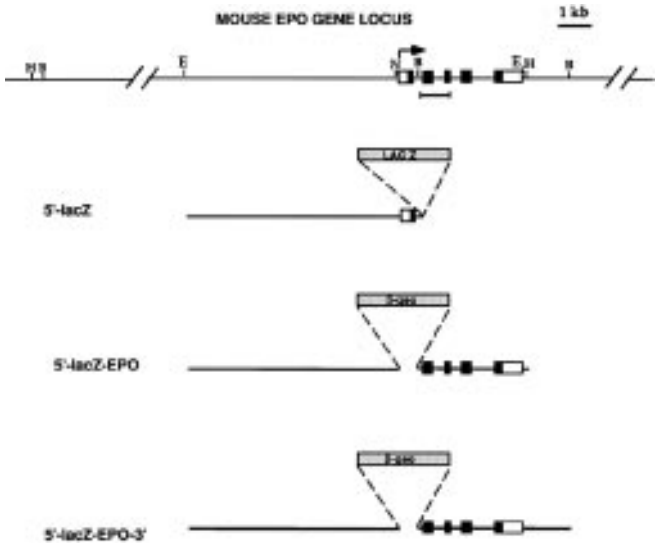

Figure 1. Schematic representation of the mouse *EPO* gene locus and the *EPO–lacZ* constructs used for generating transgenic mouse lines. The map of the mouse *EPO* gene shown at the top was derived from the mouse *EPO* gene maps described before (20,21,23). In the constructs 5′-*lacZ–EPO* and 5′-*lacZ–EPO*-3′ the *lacZ* sequence comes from the β-*geo* gene (22). The five exons (E1–E5) are presented as solid boxes. The open boxes represent 5′- and 3′-untranslated regions. The initiation of transcription begins from the 5′ *EPO* gene sequence for all the three *EPO–lacZ* constructs. The transcription initiation site of the *EPO* gene as described before is indicated by an arrow (21). The 1.0 kb *Bam*H1–*Hinc*II fragment used as a probe for Southern blot analysis of DNA is indicated by a solid bar underneath the genomic map of the mouse *EPO* gene. B, *Bam*HI; E, *Eco*RI; N, *Nar*I; H, *Hin*dIII.

ATGG) located ~360 bp downstream of the previously described hypoxia-responsive enhancer element (11) within this 1.2 kb 3′-flanking sequence.

MATERIALS AND METHODS

Plasmid constructs

The plasmid pSG5/EPO has been described before and was constructed by inserting the *Eco*RI fragment of the phage clone 60a (19–21). In the 5′-*lacZ* construct the *lacZ* gene was placed downstream of the 7.0 kb *Eco*RI–*Bam*HI fragment and has been described before (19). The 6.2 kb *Eco*RI–*Nar*I and 3.7 kb *Bam*HI–*Eco*RI fragments from the pSG5/EPO plasmid were inserted upstream and downstream respectively of the β-*geo* gene into the pSAβgeo plasmid (a generous gift from Dr Philippe Soriano, Baylor College of Medicine, Houston, TX) (22) to construct the 5′-*lacZ–EPO* plasmid. The *Nar*I and *Bam*HI sites are located 548 bp upstream and 218 bp downstream of the transcription initiation site ATG respectively (20). The *Nar*I–*Bam*HI fragment, which harbors the translational initiation site of the *EPO* gene, is missing from both the 5′-*lacZ–EPO* and 5′-*lacZ–EPO*-3′ constructs and uses the translational initiation site of the β-*geo* gene. 5′-*lacZ–Epo*-3′ (Fig. 1) was constructed by inserting the 1.2 kb *Eco*RI–*Bam*HI fragment from phage 18.c at the 3′-end of the 5′-*lacZ–EPO* construct (23).

Generation of transgenic mice

The 5′-*lacZ*, 5′-*lacZ–EPO* and 5′-*lacZ–EPO*-3′ constructs were digested with *Not*I and the respective *EPO–lacZ* fragments were purified from the agarose gel using a Gene Clean II kit (Bio 101 Inc., La Jolla, CA). Fertilized eggs were harvested from Swiss Webster Rockefeller (SWR) female mice after superovulation and mating with SJL mice. We then microinjected 2–5 pl of a 30 ng/μl solution of *EPO–lacZ* DNA fragments into the male pronucleus, as described previously (24). The injected eggs were incubated in Whitten's medium at 37°C and transferred on the same day into the oviduct of a CD-1 pseudopregnant foster mother. At least four transgenic mice lines of each construct were generated and three lines were analyzed.

DNA analysis

To identify the transgenic mice, tail DNA from 3–4-week-old pups was digested with *Hin*dIII and analyzed by Southern blot analysis using a 1 kb *Bam*HI–*Hinc*II fragment as a probe (indicated by a solid bar underneath the *EPO* gene map in Fig. 1).

Hypoxia and anemia induction

Mice were made hypoxic and anemic as described previously (19). Briefly, for hypoxia, mice were exposed to CO_2 for 3–4 min every 24 h for 5 days and the mice killed 10 min following the last CO_2 exposure. Anemia was induced by first anesthetizing the mice with a mixture of ketamine hydrochloride and xylazine and then withdrawing 0.03 ml blood via cardiac puncture. Mice were bled again 12 h later and killed 6 h following the third bleeding.

Histology

Liver, kidney, heart, lung, spleen, muscle, brain and intestine of transgenic mice were stained with X-gal, as previously described (19).

β-Galactosidase assay using *O*-nitrophenyl-β-D-galactopyranoside (ONPG)

Kidney extract (100 μg) or liver extract (1000 μg) were incubated with the ONPG (Sigma, St Louis, MO) substrate at 37°C for 16 h and the absorbance was measured at 420 nm as described elsewhere (19,25). Protein extracts of normal kidney and liver and bovine serum albumin (BSA) were used as negative controls.

Preparation of nuclear extract and electrophoretic mobility shift assay (EMSA)

Adult liver, adult kidney and fetal liver nuclear extracts were prepared as described previously (26,27). EMSA was done as described (27,28) in 25 μl binding mixture containing a 5′-^{32}P-end-labeled DNA fragment, 5 μg poly(dI·dC) (Boehringer Mannheim, Germany) and 5 μg nuclear extract in 10 mM Tris–HCl, pH 7.1, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 0.1% Triton X-100, 5% glycerol, 80 mM NaCl and 3 mM $MgCl_2$. This mixture was incubated at 4°C for 30 min and then loaded onto a 4% polyacrylamide gel (acrylamide:bis 30:1) in 0.5× TBE buffer. For competition experiments, 100 ng unlabeled oligo-duplexes were added at the beginning of the binding reaction (27).

DNase I footprint analysis

Binding reactions (50 μl) were set up using the 5′-^{32}P-end-labeled DNA fragment and nuclear extract as described for EMSA. After incubation, the mixture was digested with 4 μl 0.2 U/μl DNase I for 3 min at 20°C. The reaction was stopped by addition of 4 μl 50 mM EDTA. The mixture was then electrophoresed on a 4% polyacrylamide gel as described for EMSA. The gel was

electroblotted onto a NA45 DEAE membrane (Schleicher & Schuell Inc., Keene, NH). Free DNA (free probe) and protein–DNA complexes (gel retarded bands) were cut out from the membrane and eluted by incubating in a buffer containing 20 mM Tris, pH 8.0, 1 mM EDTA and 1.5 M NaCl at 65°C for 15 min. The eluted DNA was precipitated with 2 vol. ethanol and loaded onto a sequencing gel. Equal amounts of radioactivity of free DNA and DNA–protein complexes were loaded onto an 8% polyacrylamide sequencing gel. The G, A, T and C sequencing reactions of the DNA fragment +567/+698 were generated using a 5′-most primer (+567/+589) and AmpliCycle sequencing kit (Perkin Elmer-Cetus, Branchburg, NJ) and loaded alongside.

UV cross-linking and determination of molecular weight

The ^{32}P-end-labeled +588/+616 oligoduplex and nuclear extracts from adult kidney, adult liver or fetal liver were separately incubated and electrophoresed as described for EMSA. Following electrophoresis, the wet gel was exposed to 254 nm UV light for 30 min. After UV cross-linking the gel was exposed to X-ray film. The regions corresponding to the protein–DNA complexes (F1–F4) were cut out and sliced into small pieces and incubated with 1× SDS–PAGE sample buffer (10% glycerol, 0.25% SDS, 62.5 mM Tris, pH 6.8, 2.5% β-mercaptoethanol) containing 0.2 M NaCl at 37°C for 2 h and at 95°C for 2 min and filtered through a glass wool column. The filtrate was counted for radioactivity and equal amounts of each filtrate were loaded onto a 14% SDS–PAGE gel along with the protein molecular weight standards (Sigma, St Louis, MO).

RESULTS

Generation and characterization of transgenic mice

Figure 1 is a schematic representation of the *EPO–lacZ* gene constructs used for generating transgenic mice. At least three to four transgenic lines were generated for each of the constructs and analyzed for integration of the *EPO–lacZ* fragment by Southern blot analysis (data not shown). Table 1 shows the X-gal staining followed by visual examination of different tissues of transgenic mice carrying the 5′-*lacZ*, 5′-*lacZ–EPO* and 5′-*lacZ–EPO*-3′ constructs. X-Gal staining was detected only in kidneys, livers and intestine of transgenic mice, but not in any other tissues (heart, lung, brain, spleen and muscle) examined. However, X-gal staining was also detected in the intestine of normal non-transgenic mice, but not in any other normal tissues, suggesting that X-gal staining of intestine is non-specific and not due to transgene expression.

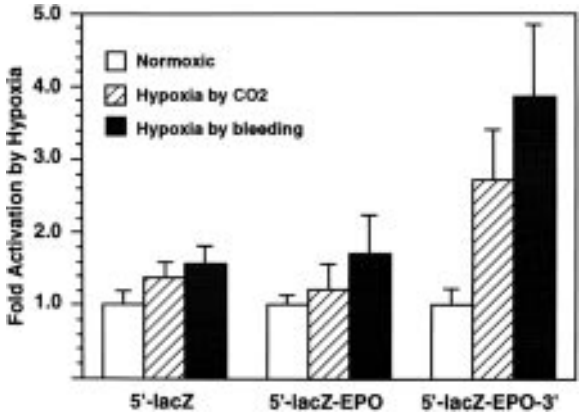

Figure 2. Relative expression of *lacZ* in the kidney of transgenic mice carrying chimeric *EPO–lacZ* constructs under normoxia, hypoxia by CO_2 and hypoxia by bleeding. Kidney extracts (100 µg) from six transgenic mouse (at least one mouse per three to four transgenic lines) were assayed for β-galactosidase enzyme expression using ONPG as substrate. The bar graph represents mean ± SD. For detail see Materials and Methods.

Hypoxia responsiveness of *EPO–lacZ* gene constructs in adult kidney

Figure 2 shows hypoxia induction of *lacZ* expression by CO_2 or by bleeding in the adult kidney of transgenic mice carrying the 5′-*lacZ*, 5′-*lacZ–EPO* and 5′-*lacZ–EPO*-3′ constructs. Hypoxia-induced activation of *lacZ* expression in kidney was relatively lower in transgenic mice lines carrying the 5′-*lacZ* (1.4- to 1.6-fold) or the 5′-*lacZ–EPO* (1.2- to 1.7-fold) constructs. This result suggests that whether *lacZ* was placed downstream of the 7.0 kb 5′ sequence (consisting of the 6.5 kb 5′-flanking sequence, exon 1 and 200 bp of intron 1) or inserted downstream of the 6.5 kb 5′-flanking sequence (substituting exon 1 and 200 bp of intron 1 of the *EPO* gene) they do not differ significantly in their hypoxia/anemia responsiveness in the adult kidney. However, the transgenic mouse lines carrying 5′-*lacZ–EPO*-3′ showed a 2.7- to 3.9-fold activation of *lacZ* expression by hypoxia/anemia when compared with *lacZ* expression under normoxic conditions.

Table 1. X-Gal staining of the various tissues of *EPO–lacZ* transgenic mouse lines

Transgenic line		*lacZ* expression							
		Kidney	Liver	Heart	Lung	Brain	Spleen	Muscle	Intestine
5′–*lacZ*									
	1	+	+	–	–	–	–	–	+
	2	+	+	–	–	–	–	–	+
	3	+	+	–	–	–	–	–	+
5′-*lacZ–EPO*									
	1	+	+	–	–	–	–	–	+
	2	+	+	–	–	–	–	–	+
	3	+	+	–	–	–	–	–	+
5′-*lacZ–EPO*-3′									
	1	+	–	–	–	–	–	–	+
	2	+	–	–	–	–	–	–	+
	3	+	–	–	–	–	–	–	+

+, X-gal staining; –, no X-gal staining.

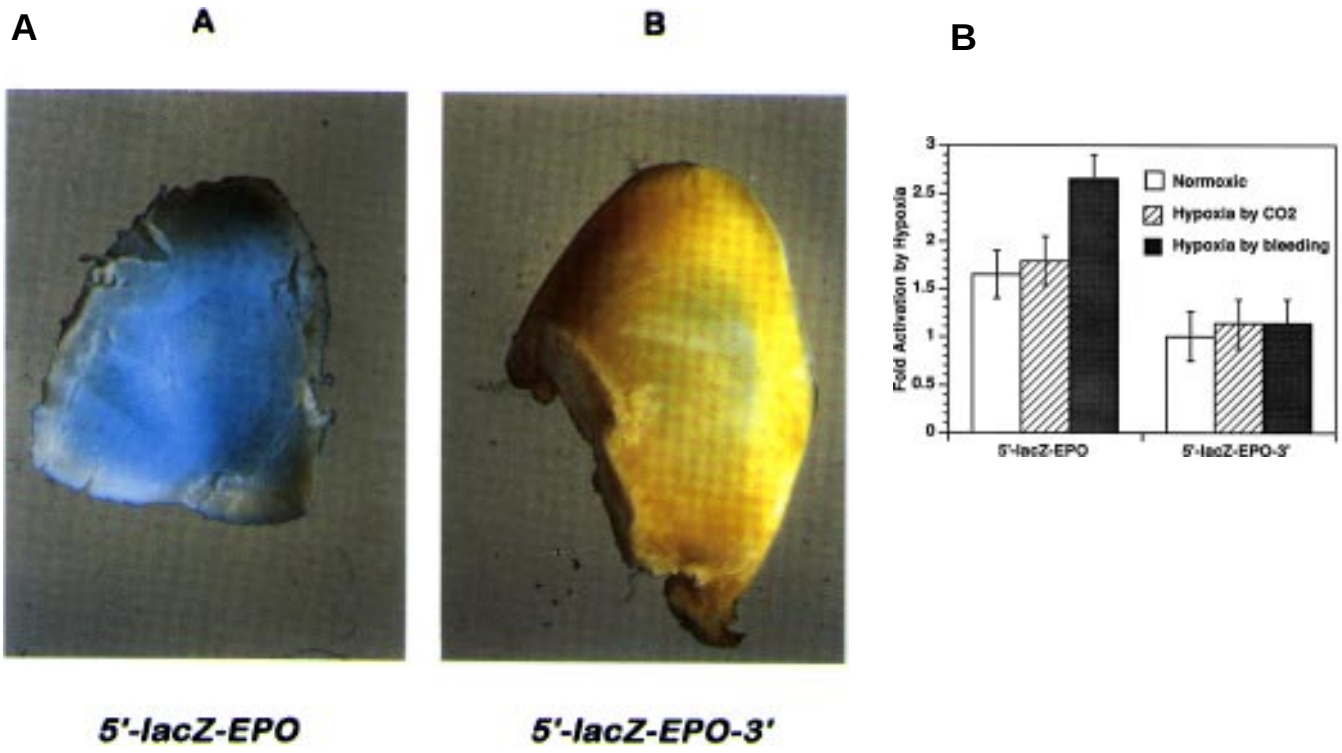

Figure 3. (**A**) X-Gal staining of the adult liver of transgenic mice carrying 5′-*lacZ–EPO* (A) and 5′-*lacZ–EPO*-3′ (B) constructs following anemia induction. Livers of anemic mice carrying 5′-*lacZ–EPO* and 5′-*lacZ–EPO*-3′ were stained simultaneously in X-gal solution as described previously (19). (**B**) Relative expression of *lacZ* in the liver of transgenic mice carrying 5′-*lacZ–EPO* or 5′-*lacZ–EPO*-3′ under normoxia, hypoxia by CO_2 and hypoxia by bleeding. Liver extracts from three to six transgenic mouse lines (at least one mouse per transgenic lines) were assayed for β-galactosidase enzyme expression using ONPG as substrate. Bar graph represents mean ± SD. For detail see Materials and Methods.

Differential expression of *lacZ* in the adult liver of transgenic mice carrying the 5′-*lacZ–EPO* and 5′-*lacZ–EPO*-3′ constructs

Figure 3A is a representative picture of X-gal staining of anemic liver of transgenic mice carrying the chimeric *EPO–lacZ* constructs without (5′-*lacZ–EPO*) or with (5′-*lacZ–EPO*-3′) the 1.2 kb 3′-flanking sequence. Transgenic mice carrying the 5′-*lacZ–EPO* construct showed a high level of *lacZ* expression in liver, whereas in the liver of mice carrying the 5′-*lacZ–EPO*-3′ construct there was only a trace amount of *lacZ* expression following anemia induction. Figure 3B shows the relative levels of *lacZ* expression (as estimated by β-galactosidase enzyme activity using ONPG as substrate) in the liver of transgenic mice carrying the 5′-*lacZ–EPO* and 5′-*lacZ–EPO*-3′ constructs under normoxia, hypoxia by CO_2 and hypoxia by bleeding conditions. *lacZ* expression in the liver of transgenic mice carrying the 5′-*lacZ–EPO*-3′ construct is very low under normoxic conditions (and is not detectable by *lacZ* staining; see Table 1) and its inducible expression in response to hypoxia and bleeding is not significant. However, livers of transgenic mice carrying the 5′-*lacZ–EPO* construct (where the 1.2 kb 3′-flanking sequence is absent) showed a relatively higher level of *lacZ* expression under normoxic conditions and a significant level of induction in response to bleeding and to a lesser extent in response to hypoxia by CO_2. Livers of transgenic mice carrying 5′-*lacZ* as described previously (19) showed a relatively low level of *lacZ* expression under hypoxia induction by CO_2 when compared with *lacZ* expression under hypoxia by bleeding. However, kidneys of transgenic mice carrying the 5′-*lacZ–EPO*-3′ construct showed relatively higher levels of *lacZ* expression under normoxic conditions and after induction by hypoxia with CO_2 or bleeding (see Fig. 2). Taken together these results suggest that the 1.2 kb 3′-flanking sequence appears to possess an adult liver-specific silencer element.

Protein factor interaction with the 1.2 kb 3′-flanking sequence of the *EPO* gene

The lack of induction of *lacZ* expression in response to hypoxia/anemia in the adult liver of transgenic mice carrying the *EPO–lacZ* construct that contains the 1.2 kb 3′-flanking sequence suggests that this region appears to harbor a regulatory element(s) that, through interactions with the liver-specific protein factor(s), may suppress induction of *EPO* gene expression. To test this possibility, we studied the 3′-flanking sequence downstream of the hypoxia-responsive enhancer element described earlier (11) for protein factor(s) interactions using nuclear extracts of adult liver, fetal liver and adult kidney on an electrophoretic mobility shift assay (EMSA). We identified a DNA segment, +567/+657, in the 1.2 kb 3′-flanking sequence [numbering with reference to the poly(A) site as +1] that interacts with multiple sets of protein factors in a tissue-specific fashion. Nuclear extracts of adult liver (F1 and F2), fetal liver (F2–F4), adult kidney (F1–F3) and adult brain (F1) formed different sets of DNA–protein complexes (Fig. 4). The F1 complex is formed at a relatively higher level than the F2 complex with the adult liver nuclear extract. Fetal liver nuclear extract does not form the F1 complex, but does form the F2–F4

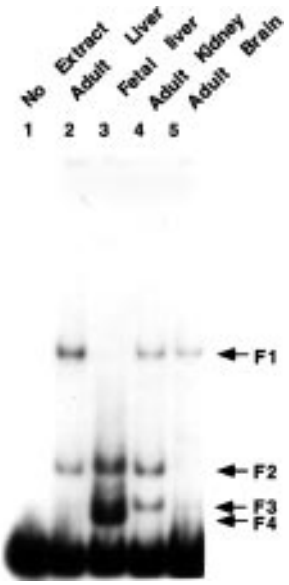

Figure 4. Electrophoretic gel mobility shift assay (EMSA). The ^{32}P-labeled +567/+657 [numbering with reference to the poly(A) site as +1] DNA fragment was incubated with 5 µg nuclear extract and 5 µg poly(d I·dC) for 30 min at 4ºC under the conditions described in Materials and Methods. The source of nuclear extract used in the binding mixture was indicated at the top of each lane.

complexes at a relatively higher level when compared with the adult liver nuclear extract. The F1 complex in adult kidney nuclear extract is formed at a relatively lower level than the F2 and F3 complexes.

These results suggest that in the adult liver in which *EPO* induction in response to hypoxia is relatively low, the F1 complex is formed at a relatively higher level when compared with the F2 complex. However, in the adult kidney and fetal liver, where *EPO* is induced in response to hypoxia, the F1 complex is formed at a relatively lower level or not at all when compared with the F2–F4 complexes. Non-EPO-producing tissues, such as adult brain and adult heart, formed only the F1 complex (data not shown).

To precisely define the sequences involved in the protein factor interactions, four oligoduplexes spanning the +567/+657 region were synthesized and included in the binding reaction for competition with the +567/+657 probe in EMSA. Unlabeled oligoduplexes made within the +567/+657 DNA segment (+567/+589, +588/+616, +610/+635 and +627/+653) were added in the binding reaction to check their capacities to compete with the probes for protein factor interactions (Fig. 5A). EMSA using oligoduplex +610/+635 is not shown. All the electrophoretic mobility shifted bands were competed off by the oligoduplex +588/+616, but not by any of the other three oligoduplexes, suggesting that the sequence within the oligoduplex +588/+616 is involved in the DNA–protein interactions.

To further confirm the above observation we ^{32}P-end-labeled the +588/+616 oligoduplex and used it as a probe instead of the +567/+657 DNA segment and competed it with the unlabeled oligoduplex +588/+616 and two other unrelated oligoduplexes (non-specific oligoduplexes 1 and 2) in an EMSA (Fig. 5B). The number and patterns of electrophoretic mobility shifted DNA–protein complexes of fetal liver, adult liver and adult kidney and the relative capacities of the unlabeled oligoduplexes for competition

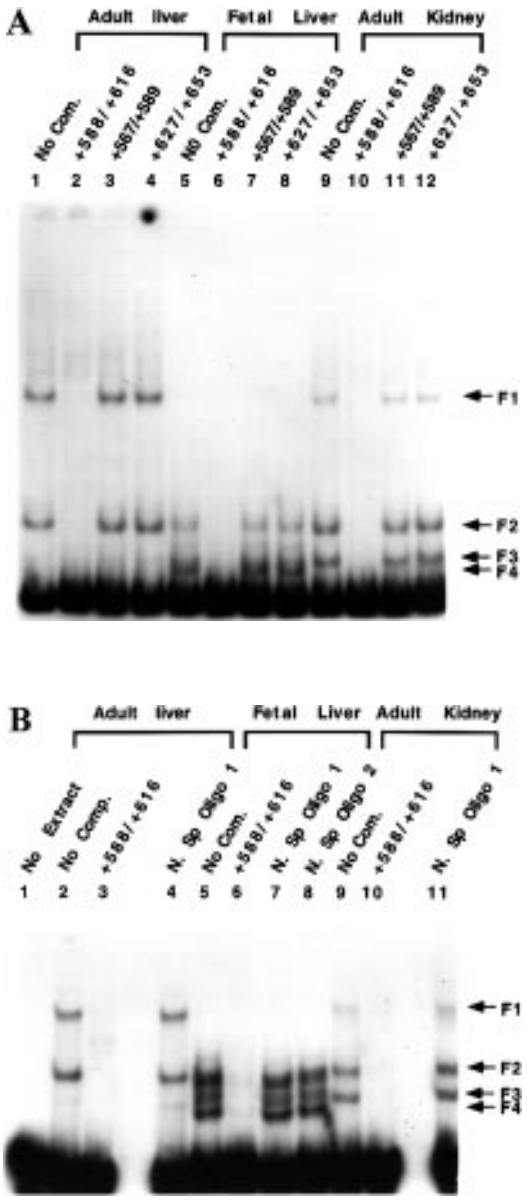

Figure 5. Competition with unlabeled oligoduplexes in EMSA. (**A**) The ^{32}P-labeled +567/+657 DNA fragment was used as a probe. (**B**) The ^{32}P-labeled +588/+616 oligoduplex was used. The nuclear extracts used in the binding reaction and the oligoduplexes used for competition are indicated at the top of each lane. EMSA was done using 5 µg nuclear extract under the conditions described in Figure 4 and Materials and Methods. For competition, 100 ng unlabeled oligoduplexes were added in the binding reactions as indicated at the top of each lane. Non-specific (NS) oligoduplexes 1 and 2 are oligoduplexes of a 25–28 bp sequence having no homology with the +588/+616 segment.

with the probes +567/+657 and +588/+616 are similar (compare Fig. 5A and B), further confirming that the sequence involved for protein factor interaction lies within the 29 bp +588/+616 sequence.

Figure 6A shows DNase I footprint analysis using nuclear extract from adult kidney and the 5′-^{32}P-end-labeled +567/+698 DNA fragment. All three DNA–protein complexes (F1–F3) showed the DNase I footprint within a 10 bp sequence +598-TCAAAGATGG-+607 when compared with the adjacent lane loaded with free DNA (free probe). A similar DNase I footprint was also detected spanning the same 10 bp sequence

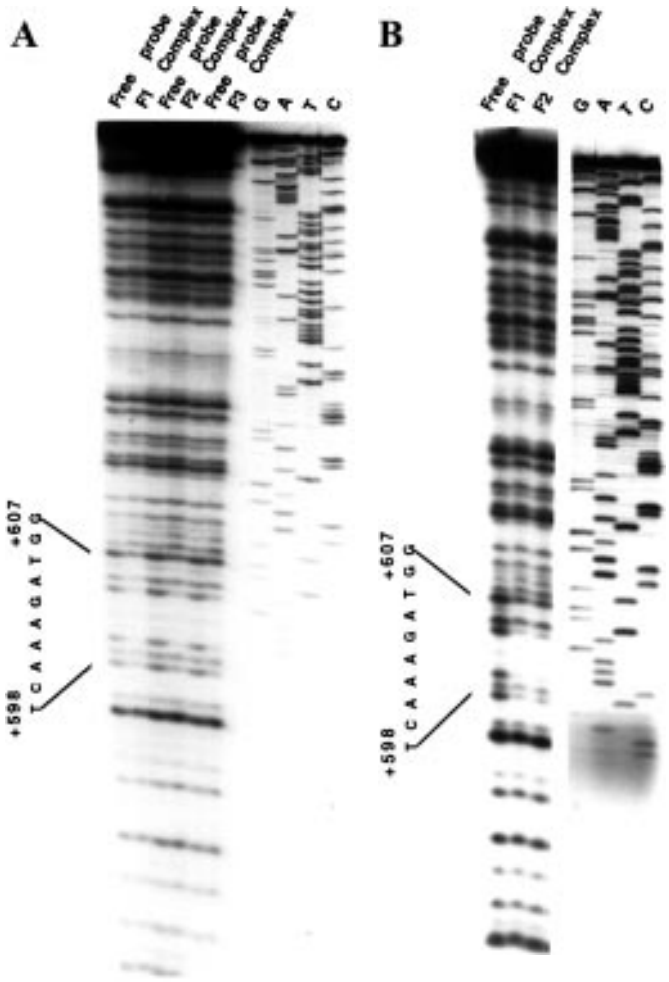

Figure 6. DNase I footprinting analysis using a 5′-end-labeled DNA fragment (+567/+698) and nuclear extracts from adult kidney (**A**) and adult liver (**B**). 5′-End-labeled DNA fragment +567/+698 was incubated with the nuclear extracts under the conditions described for EMSA and then digested with DNase I and loaded onto a 4% acrylamide gel. Following electrophoresis the gel was transferred to a DEAE membrane and the bands corresponding to F1, F2 and F3 DNA–protein complexes and free DNA (free probe) were cut out from the membrane and eluted with 20 mM Tris, pH 8.0, 1 mM EDTA, 1.5 M NaCl. The DNA was precipitated and boiled in 90% formamide-containing buffer and loaded onto a 8% sequencing gel. G, A, T and C cycle sequencing reactions were loaded alongside.

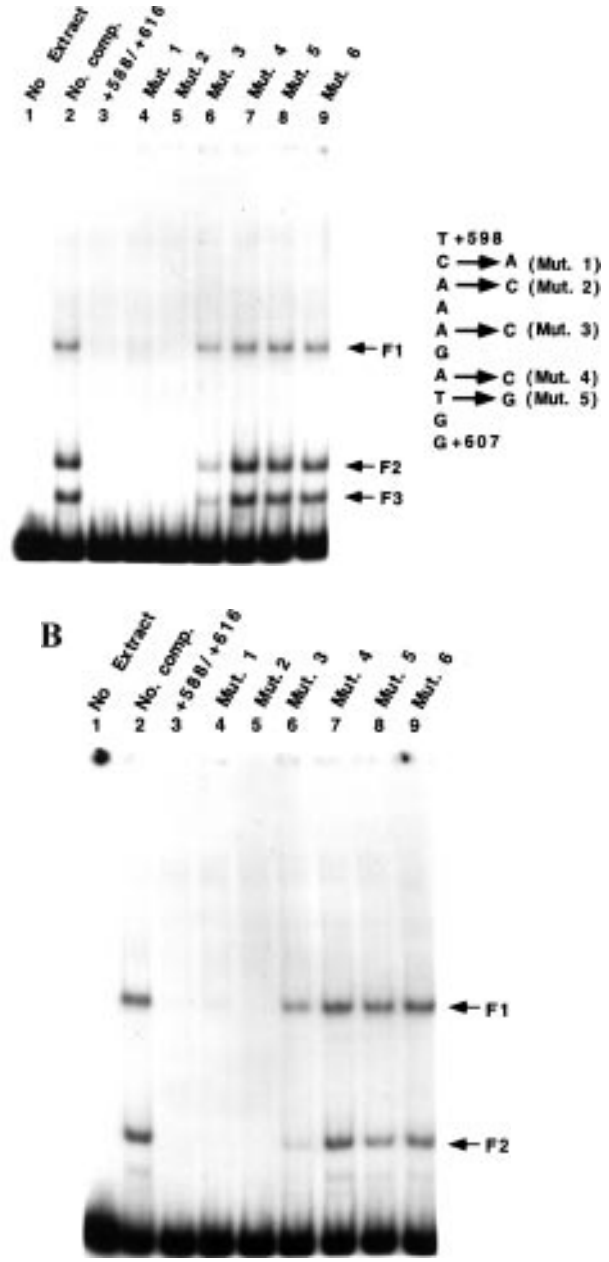

Figure 7. Competition with point-mutated oligoduplexes in EMSA using nuclear extract from adult kidney (**A**) and adult liver (**B**). The wild-type oligoduplex (+588/+616) and the mutated oligoduplexes (m1–m6) used for competition are indicated at the top of each lane. Position of the mutation in the oligoduplex is indicated on the right hand side of the figure in (A). The m6 oligoduplex is a double mutant (C→A at position 599 and T→G at positions 605).

using nuclear extracts from adult liver (Fig. 6B) and fetal liver (data not shown). These footprint data narrowed the binding sequence to a 10 bp sequence (+598/+607) and also suggested that these sets of protein factors may compete for binding to the same sequence element.

To pinpoint the bases involved in protein factor interactions, point-mutated oligoduplexes within the +588/+616 sequence were synthesized and added to the binding reaction to test their abilities to compete with the ^{32}P-end-labeled probe +567/+657 for protein factor interactions. Figure 7 shows an EMSA done in the presence of the mutated oligoduplexes. Competition data with nuclear extracts from adult kidney (Fig. 7A), adult liver (Fig. 7B) and fetal liver (data not shown) showed that a mutation in the bases +604 and +605 that lie within the footprint region (+598-TCAAAGATGG-+606) dramatically affected the capacities of the oligoduplexes to compete with the probe for protein factor interaction, suggesting that these two bases are critical for the DNA–protein interaction to occur. However, mutation of the base +602 in mutant oligoduplex 3 partially affected competition with the probe.

To estimate the approximate molecular weights of the protein factors interacting with the +588/+616 DNA segment, UV cross-linking of the DNA–protein complexes was done and analyzed by 14% SDS–PAGE (Fig. 8). The F1 complex (from adult liver and kidney) showed two protein–DNA complexes of

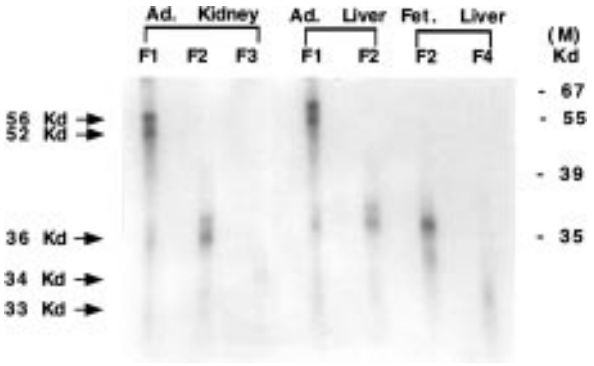

Figure 8. Determination of molecular weights by UV cross-linking coupled with SDS–PAGE analysis. The ^{32}P-labeled +588/+616 oligoduplex was incubated with nuclear extract from adult kidney, adult liver and fetal liver and electrophoresed under the conditions described for EMSA. Following electrophoresis the wet gel was UV irradiated (254 nm) for 30 min and exposed to X-ray film. The bands corresponding to F1, F2, F3 and F4 complexes from different lanes were cut out from the gel and the protein–DNA complexes were extracted and analyzed by 14% SDS–PAGE under the conditions described in Materials and Methods. The protein–DNA complexes loaded in each lane are indicated at the top. The positions of the protein molecular weight standards are indicated on the right and the approximate molecular weights of the DNA–protein complexes are indicated by an arrow on the left hand side of the figure.

~52 and 56 kDa. As the 52 and 56 kDa proteins were found in replicate experiments to exist in an equal molar ratio in the F1 complex, it is possible that heterodimerization of the 52 and 56 kDa proteins is necessary to create the binding domain for interaction with the 10 bp sequence. The other possibility would be that the 52 and 56 kDa proteins bind separately with the +588/+616 DNA probe and the resultant DNA–protein complexes run at a similar electrophoretic mobility in EMSA. The F2 protein–DNA complex of adult kidney, adult liver and fetal liver was composed of a major 36 kDa protein. In addition, the F2 complex of adult kidney and liver was also found to be accompanied by another protein at a relatively lower concentration located just above the 36 kDa protein band. The F3 (adult kidney) and F4 (fetal liver) protein–DNA complexes were 34 and 33 kDa respectively. Although equal amounts of radioactivity were loaded for all the protein–DNA complexes (F1–F4), the band intensities of the F3 and F4 complexes were relatively weak compared with the F1 and F2 complexes. This result suggests that the F3 and F4 protein–DNA complexes were not as efficiently cross-linked as those of the F1 and F2 complexes.

DISCUSSION

Analysis of *lacZ* expression in transgenic mice lines carrying *EPO–lacZ* constructs and DNA–protein interaction studies of the 1.2 kb 3′-flanking sequence suggest that: (i) the 6.5 kb 5′ sequence, the body of the *EPO* gene and 1.2 kb 3′-flanking region contain sufficient *cis*-acting sequences for tissue-specific expression of the *EPO* gene; (ii) the 1.2 kb 3′-sequence appears to possess a silencer sequence capable of suppressing hypoxia induction of *EPO* in the adult liver; (iii) a 10 bp sequence, TCAAAGATGG, located downstream of the previously characterized hypoxia-responsive enhancer element (11) and within the 1.2 kb 3′ region interacts with different sets of protein factors from adult liver, adult kidney and fetal liver nuclear extracts.

Maxwell *et al.* (12), using a transgenic mouse model, reported earlier that *cis*-acting sequences that regulate renal *EPO* lie within 1.5–9 kb 5′ and 3.5 kb 3′ of the mouse *EPO* gene. Semenza and colleagues (10,29,30) studied the *cis*-regulatory elements in the 5′- and 3′-flanking sequences of the human *EPO* gene by generating transgenic mice carrying 0.4, 6.0 or 14 kb of the 5′-flanking sequences and 0.7 kb of the 3′-flanking sequence. These transgenic mouse models suggested that the *cis*-acting sequences involved in hepatic and renal expression are different. The expression of *EPO* in the kidney appeared to be dependent on the sequences between 6.0 and 14.0 kb 5′ of the human gene. A recent report by Madan *et al.* (31) also suggested that for appropriate tissue-specific expression of the human *EPO* gene, at least a 9.5 kb 5′-flanking sequence and an 8.5 kb 3′-flanking sequence are required.

Our transgenic mice, which carry the 6.5 kb 5′-flanking sequence, the body of the *EPO* gene and the 1.2 kb 3′-flanking sequence, express *lacZ* at a higher level following hypoxia/anemia induction in the adult kidney than in the adult liver. However, *lacZ* is induced following hypoxia/anemia induction in both the adult kidney and liver of transgenic mice that carry the construct without the 1.2 kb 3′-flanking region (5′-*lacZ–EPO*). These results suggest that the 1.2 kb 3′-flanking region of the mouse *EPO* gene appears to contain a sequence element that may be involved in suppression of *EPO* gene induction in the adult liver in response to hypoxia/anemia.

Transgenic mice generated with additional 5′- and 3′-flanking sequences (9.0 kb 5′-flanking, the body of the gene and 3.5 kb 3′-flanking sequence) of the mouse *EPO* gene showed induction in liver following anemia induction (12). It is possible that sequence downstream of the 1.2 kb 3′ mouse *EPO* sequence may harbor an additional enhancer element(s), which through interaction of protein factors induced under anemic conditions can act in concert with the 3′-proximal hypoxia-responsive enhancer element (11) to overcome suppression of the silencer sequence present in the 1.2 kb 3′ region.

lacZ expression in response to hypoxia and bleeding is 1.4- to 1.6-fold, 1.2- to 1.7-fold and 2.2- to 3.9-fold when compared with the normoxic level of *lacZ* expression in the kidneys of transgenic mice carrying 5′-*lacZ*, 5′-*lacZ–EPO* and 5′-*lacZ–EPO*-3′ constructs respectively. The fold activation of *lacZ* in response to hypoxia/anemia is relatively lower than that reported for induction of endogenous *EPO* expression and intact *EPO* transgene expression (50- to 100-fold) in response to hypoxia (32). However, a recent report indicated that transcriptional induction of *EPO* mRNA accounts for only a 10-fold increase and post-transcriptional stabilization of *EPO* mRNA appears to be the major mechanism of hypoxic induction of EPO (32). It is therefore possible that the hybrid *lacZ–EPO* mRNA is not as stable as that of *EPO* mRNA and could account for the differences in hypoxia induction. This discrepancy in induction could also be due to the presence of an additional hypoxia-responsive enhancer element(s) outside the boundary of the 6.5 kb 5′- and 1.2 kb 3′-flanking sequences of the mouse *EPO* gene used for making the *EPO–lacZ* constructs.

By analyzing the 1.2 kb 3′-flanking sequence, we identified a DNA segment, +567/+657 [numbering with reference to the poly(A) site as +1], downstream of the previously described hypoxia-responsive enhancer element (11) that forms multiple sets of protein–DNA complexes in EMSA using nuclear extracts from adult liver (F1 and F2 complexes), adult kidney (F1–F3 complexes), fetal liver (F2–F4 complexes) and adult brain (F1

complex only). The capacity of the 10 bp *EPO* sequence to bind multiple protein factors tissue and development specifically suggests that the relative abundance and competition of these protein factors for binding the sequence element may play a role in *EPO* gene expression. The 52 and 56 kDa protein factors that form the F1 complex may be responsible for silencing the *EPO* gene in the adult liver. The 36, 34 and 33 kDa proteins that form the F2–F4 complexes respectively may compete with the 52 and 56 kDa proteins and may prevent silencing of *EPO* gene expression in the adult kidney and fetal liver. The F3 (34 kDa) and F4 (33 kDa) complexes may act as enhancers or may act by preventing the formation of the F1 silencer complex.

The role of silencer elements has also been documented in other genes: in a tissue-specific position effect on alcohol dehydrogenase expression in *Drosophila melanogaster* (33); in repression of neuronal gene transcription in non-neuronal cells through interaction of the neuron-restrictive silencer factor (NRSF) with the neuron-restrictive silencer element (NRSE) (34); in the role of multiple silencer elements in the regulation of the chicken vimentin gene (35). A silencer and an enhancer element have been reported in the 3′-flanking region of the chicken and duck α-globin gene, similarly to the *EPO* gene (36). We did not find any homology of the 10 bp sequence element (TCAAAGATGG) to any of the silencer elements reported in other genes (33–36).

The hepatoma cell lines HepG2 and Hep3B were shown to regulate EPO production in culture in response to oxygen tension and have been successfully used in defining the hypoxia-responsive enhancer elements (11). However, appropriate kidney and liver cell models are not available to test the *cis*-element responsible for tissue- and development-specific expression of the *EPO* gene. We plan to generate transgenic mice carrying *EPO–lacZ* gene constructs that contain different deletions in the 1.2 kb 3′-flanking sequence, including the 10 bp sequence (which interacts with multiple sets of protein factors both tissue and development specifically), and containing point mutations within the 10 bp sequence to precisely define the role of this 3′-sequence in tissue- and development-specific regulation of *EPO* gene expression.

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