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Genetics

Hypoxia-inducible nuclear factors bind to an enhancer element located 3′ to the human erythropoietin gene

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ABSTRACT **Human erythropoietin gene expression in liver and kidney is inducible by anemia or hypoxia. DNase I-hypersensitive sites were identified 3′ to the human erythropoietin gene in liver nuclei. A 256-base-pair region of 3′ flanking sequence was shown by DNase I protection and electrophoretic mobility-shift assays to bind four or more different nuclear factors, at least two of which are induced by anemia in both liver and kidney, and the region functioned as a hypoxia-inducible enhancer in transient expression assays. These results provide insight into the molecular basis for the regulation of gene expression by a fundamental physiologic stimulus, hypoxia.**

In mammals, erythropoietin (EPO) is the primary humoral regulator of red blood cell production and thus of blood oxygen-carrying capacity. EPO RNA levels increase several hundredfold in rodent liver and kidney in response to anemia or hypoxia (1, 2). EPO gene expression is believed to be induced by hypoxia or anemia via a single mechanism (reviewed in ref. 3). The signal sensed by EPO-producing cells is probably a decrease in local tissue oxygen tension, whether due to decreased blood oxygen-carrying capacity (anemia) or decreased ambient oxygen concentration (hypoxia). EPO gene expression in Hep3B human hepatoma cells can be induced in 1% O_2 (4, 5), demonstrating that the same cell type can sense hypoxia and respond by increasing its steady-state level of EPO RNA. Nuclear extracts prepared from Hep3B cells cultured in 1% O_2 support a higher level of EPO gene transcription *in vitro* than extracts from cells cultured in 20% O_2 (6). EPO gene expression in Hep3B cells (4, 5) and *in vivo* (2, 7) can also be stimulated by $CoCl_2$ administration.

By introducing DNA containing the human EPO gene into the mouse genome via pronuclear microinjection, we have identified cis-acting DNA sequences that regulate tissue-specific, inducible human EPO gene expression. Transgenes of 4 kilobases (kb) (tgEPO4) and 10 kb (tgEPO10) containing the human EPO gene, a 3′ flanking region of 0.7 kb, and 5′ flanking regions of 0.4 kb and 6 kb, respectively, are inducibly expressed in adult liver but not in kidney (8, 9). In the liver of anemic tgEPO10 mice, human EPO RNA is synthesized specifically by perivenous hepatocytes and the amount of EPO RNA per cell increases as anemia is made more severe (10). When tgEPO4 or tgEPO10 mice are made anemic, human EPO RNA increases by several orders of magnitude in liver compared with the uninduced state, indicating the presence of sequences mediating inducible liver expression in close proximity to the human EPO gene (8, 9).

MATERIALS AND METHODS

DNase I-Sensitivity Studies. Nuclei were isolated and then digested with DNase I at 0, 1, 2, or 5 μg/ml for 2 min at 25°C, and DNA was isolated as described (11).

Nuclear Extracts. Liver (41 g) and kidney (12.6 g) were isolated from 21 untreated mice, kidney (7.6 g) was isolated from 15 anemic mice treated with phenylhydrazine (mean hematocrit, 24%), and liver (9.7 g) was isolated from 5 phenylhydrazine-treated mice (mean hematocrit, 21%). Phenylhydrazine was given i.p. (60 μg/g of body weight) every 12 hr starting 36 hr prior to sacrifice. Tissue was frozen in liquid nitrogen and stored at −80°C. Nuclei were isolated as described (12, 13), except that all buffers contained aprotinin, leupeptin, and pepstatin (Sigma), each at 2 μg/ml.

DNase I Footprint Analysis. A 256-base-pair (bp) sequence extending 3′ from within the human EPO gene polyadenylylation signal was amplified by polymerase chain reaction (PCR) using 5′ end-labeled primers shown in Fig. 3. Amplification was as follows: 6 min at 94°C; 30 sec at 94°C, 45 sec at 50°C, 45 sec at 72°C, for 30 cycles; and 10 min at 72°C. For footprint analysis (12, 14), PCR probe (7500 cpm; 0.4 ng) was incubated with 40 μg of nuclear extract protein and 1 μg of poly(dI-dC) (Pharmacia) in 80 μl of 10 mM Hepes, pH 7.9/10% (vol/vol) glycerol/50 mM KCl/5 mM $MgCl_2$/0.1 mM EDTA/0.75 mM dithiothreitol for 15 min on ice, followed by 2 min at 25°C, and DNase I (0.3 μg) was added for 1 min at 25°C. DNA was extracted, precipitated, resuspended in formamide, and analyzed by 8 M urea/8% PAGE.

Gel Shift Assays. Complementary oligonucleotides were synthesized with *Hin*dIII (sense strand) and *Eco*RI (antisense strand) 5′ overhangs, except OL-21, -23, and -33, which were composed entirely of EPO gene sequences. The sense strand was end-labeled using phage T4 polynucleotide kinase and [γ-^{32}P]ATP and annealed to a 10-fold excess of antisense strand. Double-stranded probe was purified by 10% PAGE and elution in 10 mM Tris·HCl, pH 7.5/50 mM NaCl. Probe ($1–2 \times 10^4$ cpm; 30–150 pg) was incubated with 1 μg of nuclear extract and 2–4 μg of poly(dI-dC) in 20 μl of 10 mM Tris·HCl, pH 7.5/12.5% glycerol/50 mM KCl/5 mM $MgCl_2$/1 mM EDTA/0.1% Triton X-100/1 mM dithiothreitol for 30 min at room temperature after preincubation of all components except probe for 15 min. After preelectrophoresis for 1 hr, 5% PAGE was performed in 50 mM Tris·HCl/380 mM glycine/2 mM EDTA, pH 8.5, at 180 V for 2 hr at 4°C. The gel was dried and autoradiographed at −80°C for 1–5 days.

Transient Expression Studies. Plasmids contained the simian virus 40 (SV40) basal promoter, bacterial chloramphenicol acetyltransferase (CAT) coding sequence, and the SV40 splice site and polyadenylylation signal (pCAT-promoter, Promega; here designated pSVcat). On the 3′ side of these sequences was either a 236-bp SV40 enhancer element (pCAT-control, Promega; here designated pSVcatSV), a 256-bp PCR product, or a 2.5-kb *Bgl* II–*Bam*HI fragment, with the latter two inserted into the *Bam*HI site of pSVcat. Plasmid DNA (15 μg) was transfected into 2.5×10^6 Hep3B cells by using 90–100 μg of Lipofectin reagent (BRL) and

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Abbreviations: CAT, chloramphenicol acetyltransferase; EPO, erythropoietin; FS, flanking sequence(s); KIE, kidney inducibility element; LIE, liver inducibility element; nt, nucleotides; SV40, simian virus 40; UTR, untranslated region.

incubated for 5 hr in reduced-serum medium (Opti-MEM I; BRL). Cells were then placed in complete medium (5) with or without 50 μM $CoCl_2$ and incubated for 24–40 hr at 37°C in a 5% CO_2/95% air incubator ("20% O_2") or a closed chamber flushed with 1% O_2/5% CO_2/94% N_2 ("1% O_2"). Cell extracts were prepared and protein content was determined (Bio-Rad protein assay). CAT protein was quantitated by ELISA (5 Prime → 3 Prime, Inc.) using standard curves generated with purified CAT, after correction for values obtained from untransfected control plates. The ratio pg of CAT/mg of protein was normalized to the value for pSV-catSV in cells cultured in 20% O_2.

RESULTS

DNase I-Hypersensitive Sites 3′ to the Human EPO Gene in Liver Nuclei. Nuclei were isolated from brain, kidney, liver, and spleen of mice carrying six tandem copies of the 10-kb *Bam*HI–*Eco*RI human EPO transgene, which is inducibly expressed exclusively in liver (9). Nuclei were treated with DNase I, and DNA was extracted and digested with restriction endonucleases. Blot hybridization was performed using a 0.65-kb *Bgl* II–*Pst* I human EPO gene probe containing 3′ untranslated region (3′-UTR) and 3′ flanking sequence (3′-FS) (8). Fig. 1, which is representative of the results obtained, shows that a 4.4-kb hybridizing restriction fragment was generated by *Bgl* II digestion in the absence of DNase I digestion. In DNA from liver nuclei, 0.6-kb and 0.5-kb fragments were also seen after DNase I digestion. The *Bgl* II site is in the 3′-UTR at nucleotide (nt) 2957 and the polyadenylylation site is at nt 3339 (15). The presence of hypersensitive sites 0.5 kb and 0.6 kb 3′ to the *Bgl* II site map to the region of nt 3460 and nt 3560 in the 3′-FS. Hypersensitive sites were not detected in kidney, brain, or spleen. Nuclei were also isolated from human adult liver and DNase I-hypersensitive sites were mapped by blot hybridization (data not shown) to a similar location 3′ to the EPO gene as was seen for the transgene in mouse liver nuclei.

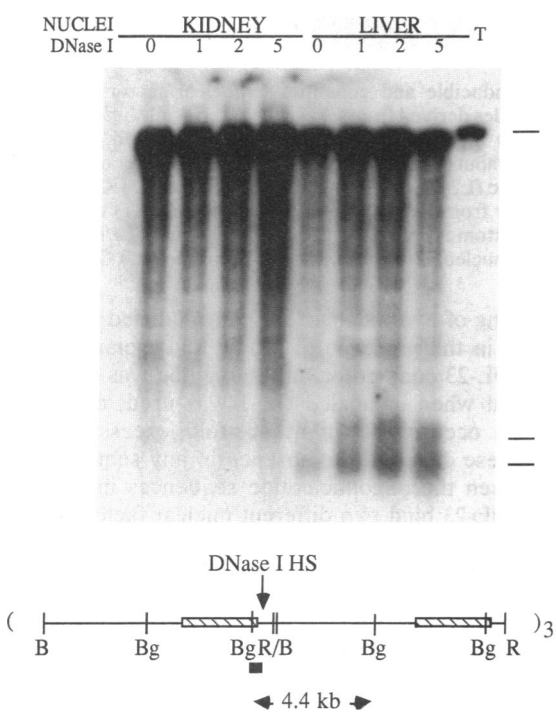

FIG. 1. Human EPO gene 3′ flanking region is hypersensitive to DNase I digestion in liver nuclei of transgenic mice. Nuclei were isolated from liver and kidney of mice carrying six tandem copies (two of which are shown) of a 10-kb *Bam*HI (B)–*Eco*RI (R) human genomic fragment containing the EPO gene (hatched box). Nuclei were treated with indicated amounts of DNase I (in μg/ml), and DNA was extracted and digested with *Bgl* II (Bg), as was DNA from a tail biopsy specimen (T). A 4.4-kb restriction fragment (horizontal arrows) was detected by the probe (dark box). Vertical arrow, location of DNase I-hypersensitive sites (HS) in liver nuclei.

Binding of Nuclear Factors 3′ to the Human EPO Gene. Three overlapping sequences spanning >600 bp 3′ to the *Bgl* II site were amplified by PCR and used as probes for DNase I footprint analysis. Only the most distal probe, containing 16 bp of 3′-UTR and 240 bp of 3′-FS (nt 3324–3579; ref. 15), included regions protected from DNase I digestion by the binding of liver nuclear factors. In Fig. 2, the 256-bp probe was 5′ end-labeled on the sense strand. Probe alone was randomly digested, generating a ladder of fragments (lane 6). Incubation of probe with nuclear extract from liver of nonanemic (L1) or anemic (L2) mice resulted in areas of protection from, and areas of hypersensitivity to, DNase I digestion. Regions B and C were protected from DNase I digestion by either extract. Regions A and D were hypersensitive (HS) to DNase I digestion in the presence of nonanemic liver extract (L1; lanes 7–9) but were protected from digestion by nuclear extract from anemic liver (L2; lanes 10–12), indicating that factors induced by anemia bind to regions A and D. Complementary regions of protection and hypersensitivity were observed when the probe was 5′ end-labeled on the antisense strand (data not shown). The forward PCR primer was also utilized as a primer for nucleotide sequence analysis (lanes 1–4 and 13–16), allowing identification of the protected sequences.

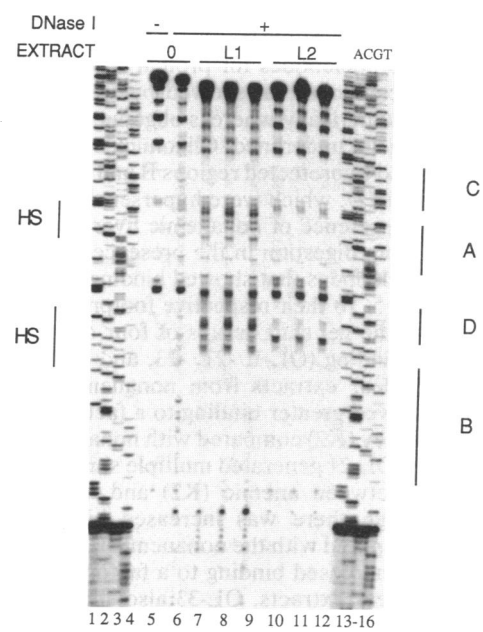

FIG. 2. DNase I footprint analysis of EPO gene 3′-flanking-region probe incubated with liver nuclear extracts. A 256-bp region was amplified by PCR with a 5′ end-labeled forward primer and used as a probe. Sequence ladders (lanes 1–4 and 13–16) represent dideoxy reactions (A, C, G, T) using the same labeled primer as in PCR. Probe was incubated with no extract (0; lanes 5 and 6) or with liver nuclear extract from nonanemic mice (L1; lanes 7–9) or anemic mice (L2; lanes 10–12) and digested with 0 (−; lane 5) or 0.3 μg (+; lanes 6–12) of DNase I. Full-length probe is at the top of each lane; smaller fragments seen in lane 5 and across gel represent either probe degradation or extraneous PCR products. Incubation of probe with L1 and L2 was performed in triplicate. HS, hypersensitive sites formed upon incubation of probe with L1 prior to digestion. A–D, regions protected from DNase I digestion after incubation of probe with L1 or L2.

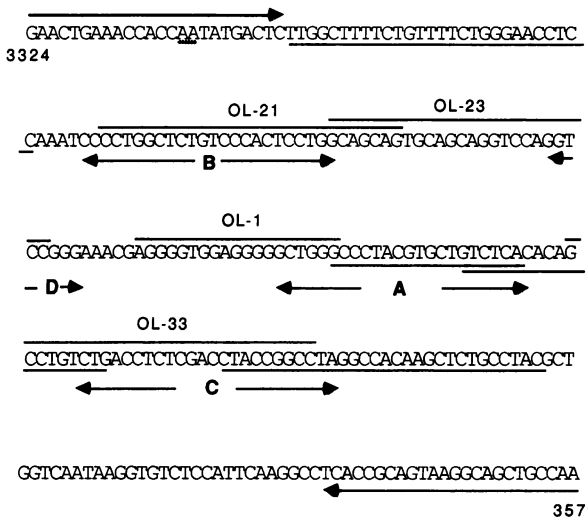

FIG. 3. Multiple binding sites for liver nuclear factors in EPO gene 3′-FS. Nucleotide sequence of 256-bp region amplified by PCR is shown, with coordinates (nt 3324–3579) based on published sequence (15) from which it differs at nt 3434, where we found C rather than T. Single arrow, single-stranded oligonucleotide used as primer for PCR and nucleotide sequence analysis (reverse primer was complementary to sequence shown); double arrow, region protected from DNase I digestion after incubation with liver extracts (see Fig. 2); underline, sequences that as double-stranded oligonucleotides did not bind to liver nuclear factors in gel shift assays; overline, sequences that bound liver nuclear factors (see Fig. 4); jagged underline, polyadenylylation site.

Fig. 3 shows the 256-bp amplified region, the regions of protection in the footprinting assay (A–D), and the results of testing eight oligonucleotides for protein binding in gel shift assays. Oligonucleotides that showed binding are overlined and designated numerically whereas oligonucleotides that did not show binding are underlined. Oligonucleotides OL-21 and OL-33 correspond to protected regions B and C, respectively. For regions A and D, which were hypersensitive to DNase I digestion in the presence of nonanemic liver nuclear extract but protected from digestion in the presence of anemic liver extract, oligonucleotides that showed binding (OL-1 and OL-23) were located 5′ to their respective footprints.

Fig. 4 shows the gel shift assays of four oligonucleotides demonstrating binding (OL-1, -21, -23, and -33), using liver and kidney nuclear extracts from nonanemic and anemic mice. OL-1 showed greater binding to a factor from anemic liver (L2) or kidney (K2) compared with nonanemic liver (L1) or kidney (K1). OL-21 generated multiple shifted bands, with no difference between anemic (K2) and nonanemic (K1) extracts, although there was increased binding with the anemic (L2) compared with the nonanemic (L1) liver extract. OL-23 showed increased binding to a factor in anemic liver and kidney nuclear extracts. OL-33 also showed binding to liver and kidney nuclear factors, with an additional band present after incubation with anemic liver and kidney extracts compared with nonanemic liver extract. Each of the four oligonucleotides produced a qualitatively different gel-shift pattern, suggesting that a different factor bound to each. OL-1, -21, and -33 were incubated with nuclear extracts from HeLa (H) and teratocarcinoma (T) cells and showed different binding patterns with these extracts than with the liver and kidney nuclear extracts.

Other oligonucleotides from the region of the PCR probe (Fig. 3) and the 3′-UTR (see below) were tested in gel shift assays and did not demonstrate specific binding to liver or kidney nuclear factors, whereas OL-1, -21, -23, and -33 showed specific binding that was inhibited by excess unlabeled homologous, but not heterologous, oligonucleotide.

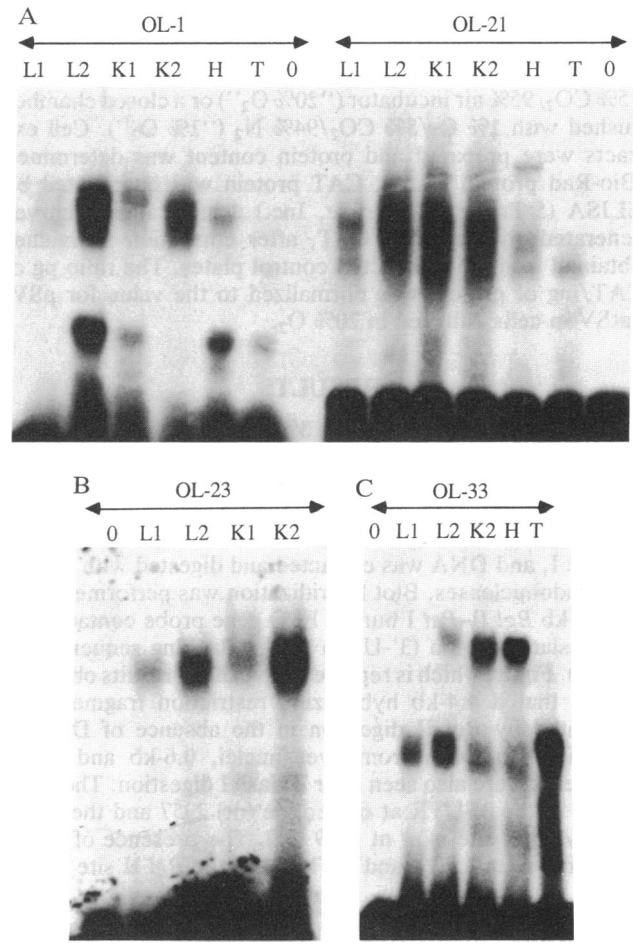

FIG. 4. Inducible and constitutive nuclear factors binding to oligonucleotides derived from EPO gene 3′-FS. Gel shift assays were performed by incubating ^{32}P-labeled double-stranded oligonucleotide probe without (0) or with nuclear extract from liver of nonanemic (L1) or anemic (L2) mice, from kidney of nonanemic (K1) or anemic (K2) mice, or from HeLa (H) or teratocarcinoma (T) cells. Radioactivity at bottom represents free probe. (*A*) OL-1 (*Left*) and OL-22 (*Right*) oligonucleotide probes. (*B*) OL-23 probe. (*C*) OL-33 probe.

When binding of liver nuclear extract to labeled OL-23 was carried out in the presence of a ≥50-fold molar excess of unlabeled OL-23 competitor, probe binding was completely blocked, but when unlabeled OL-1 was used, only partial competition occurred even at 400-fold excess (data not shown). These data and the absence of any sequence similarity between the oligonucleotide sequences indicate that OL-1 and OL-23 bind two different nuclear factors induced by anemia/hypoxia.

Additional gel shift assays were performed to define the binding site within OL-1 (Fig. 5*A*). Two sequences similar to OL-1 that are present in the 3′-UTR were synthesized as oligonucleotides (OL-7 and -9). Gel shift assays, using as probes OL-7, OL-9, and substitution mutants (OL-29 and -31) that increase the similarity between OL-1 and OL-7 or OL-9, demonstrated that the OL-1-binding activity recognized sequences other than, or in addition to, those shared by OL-1 and either OL-7 or OL-9. Overlapping oligonucleotides containing the 5′ end of OL-1 (OL-37), the 3′ end (OL-39), and the center (OL-41) were also tested, demonstrating a requirement for the 14 nt at the 5′ end of OL-1, as only OL-37 showed binding comparable to OL-1. The data in Fig. 5*A* suggest that the GGGG(G) repeats of OL-1 are necessary for binding, as exemplified by the poor binding of OL-9, which lacks two G

	Oligonucleotide Sequence	EPO Sequences	Oligo No.	Binding
A	******* *****			
	AGGGGTGGAGGGGGCTGGG	3436–3454	OL-1	++++
	GGGGCTGGCCTCTGGCTCTC	3263–3282	OL-7	+
	TTGACACCGGGGTGGTGGGA	3226–3245	OL-9	+
	AGGGGTGGAGGGGGCTGGC	OL-1 (μ3454)	OL-29	++++
	CGGGGTGGTGGGGGCTGGG	OL-1 (μ3436, 3444)	OL-31	++++
	AAACGAGGGGTGGAGGGGG	3431–3449	OL-37	++++
	GTGGAGGGGGCTGGGCCC	3440–3457	OL-39	−
	GATCGTGGAGGGGGAGATC	3440–3449	OL-41	−
B	CCTGGCTCTGTCCCACTCCTGGC	3382–3404	OL-47	++++
	CCTGGCTCTGTCCCACTCCTGGCAGCAGTGCAGCAG	3382–3417	OL-21	++++
	GCAGCAGTGCAGCAGGTCCAGGTCC	3403–3427	OL-23	++++
	GCAGCAGTGCAGCAG	3403–3417	OL-45	−
	AGGTCCAGGTCCGGGAAA	3416–3433	OL-43	−

FIG. 5. Definition of nuclear factor-binding sites. (*A*) Summary of gel shift data for OL-1 and related oligonucleotides. Seven oligonucleotides with sequences related to OL-1 were tested for binding in gel shift assays using liver nuclear extract from nonanemic mice. Sequences in common with OL-1 are underlined. Coordinates are those given in ref. 15. Binding, amount of shifted radioactivity when given oligonucleotide was used as a probe compared with OL-1 probe: −, no binding. +, slight binding. ++++, same degree of binding as OL-1. Oligonucleotides contain the following sequences: OL-1, -37, and -39 contain overlapping sequences from 3′-FS. OL-7 and -9 contain related sequences from 3′-UTR. OL-29, -31, and -41 are substitution mutants (μ) of OL-1 as indicated. Stars indicate nucleotides present in all oligonucleotides that showed binding. (*B*) Summary for OL-21, OL-23, and related oligonucleotides. OL-43, -45, and -47, which contain sequences within OL-21 or -23, were tested in gel shift assays using liver nuclear extract from nonanemic and anemic mice. Direct repeat sequences are underlined. Binding, amount of shifted radioactivity when given oligonucleotide was used as a probe compared with OL-21 or -23 probe.

residues at its 3′ end, and of OL-39 and -41, which lack three G residues at the 5′ end.

OL-21 and -23 overlap but appeared to bind different nuclear factors. In OL-43, -45, and -47, the unique and overlapping sequences of OL-21 and -23 were isolated for analysis (Fig. 5*B*). OL-47, which contains the 5′ end of OL-21, showed binding that was completely blocked by unlabeled OL-21 (data not shown). Neither OL-45 nor OL-43, which contained the 5′ and 3′ ends of OL-23, respectively, showed binding when used as probes.

Functional Analysis of 3′-FS. The 256-bp PCR product was tested for enhancer activity in transient expression assays. The fragment was cloned 3′ to a fusion gene in which transcription of coding sequences for the bacterial enzyme CAT were driven by the SV40 promoter. The EPO sequences were inserted in the sense (pSVcatEPO-1S) and antisense (pSVcatEPO-1A) transcriptional orientation. Expression of these constructs was compared to that of plasmids containing no enhancer (pSVcat) or the SV40 enhancer (pSVcatSV) at the same location. DNA was introduced into duplicate plates of Hep3B cells, which were cultured for 24–40 hr in 20% or 1% O_2 before harvest. For each experiment, measurements of CAT were normalized to the amount of CAT produced by pSVcatSV in Hep3B cells grown in 20% O_2. Means for the normalized values from four independent transfection experiments are shown in Fig. 6 *Upper*. While pSVcatSV showed no induction of CAT expression in Hep3B cells cultured in 1% vs. 20% O_2, pSVcatEPO-1S was induced an average of 3.2-fold and pSVcatEPO-1A was induced 2.6-fold by hypoxia, to levels exceeding those of pSVcatSV. A 2.5-kb DNA fragment extending 3′ from the *Bgl* II site in the 3′-UTR, including the 256-bp PCR fragment with additional 3′-UTR and 3′-FS, was cloned into pSVcat to generate pSVcatEPO-2S and -2A and transfected into Hep3B cells in two independent experiments. pSVcatEPO-2S and -2A were induced 3.2- and 4.3-fold by hypoxia, but the absolute levels of expression were lower than those of the pSVcatEPO-1 plasmids. Hypoxia induced a 9-fold mean increase in endogenous EPO production by transfected Hep3B cells as measured by RIA (data not shown). Addition of $CoCl_2$ to the culture medium also induced expression of the pSVcatEPO-1 plasmids (Fig. 6 *Lower*).

DISCUSSION

EPO production is inversely correlated with tissue oxygen tension (16), but little is known about the molecular mechanism by which cells sense and respond to hypoxia. We have attempted to identify the DNA sequences and transcription factors responsible for induction of EPO gene expression by hypoxia in liver and kidney. Transgenic mice carrying either tgEPO4 or tgEPO10 inducibly express the human EPO gene in liver but not in kidney (9), localizing the liver inducibility element (LIE) to the 4 kb shared in common, including the

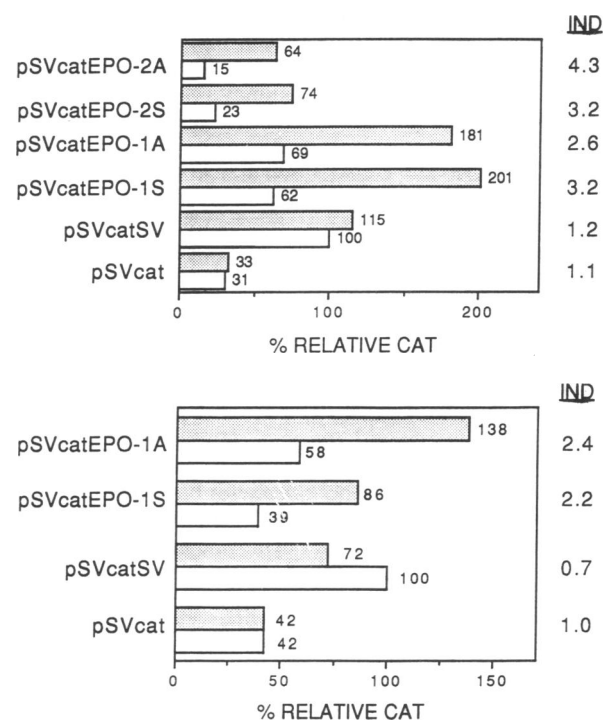

FIG. 6. EPO 3′-FS functions as a hypoxia-inducible enhancer in Hep3B cells. Plasmids contained the SV40 basal promoter fused to CAT coding sequence and, 3′ to the transcription unit, either no additional DNA (pSVcat), 256 bp (pSVcatEPO-1) or 2.5 kb (pSVcatEPO-2) of EPO gene 3′-FS in the sense (S) or antisense (A) transcriptional orientation, or the 236-bp SV40 enhancer (pSVcatSV). (*Upper*) CAT expression in transfected Hep3B cells cultured in 1% (shaded bars) or 20% (open bars) O_2. IND, inducibility (1% O_2/20% O_2). (*Lower*) CAT expression in transfected Hep3B cells cultured in the presence (shaded bars) or absence (open bars) of $CoCl_2$. IND, inducibility (+ $CoCl_2$/− $CoCl_2$).

gene with 0.4 kb of 5′-FS and 0.7 kb of 3′-FS. Mice carrying a transgene containing the 5′ 1.2 kb of tgEPO4 (5′-FS, exon 1, intron 1) fused to T-antigen coding sequence show no transgene expression (G.L.S., unpublished data), indicating that sequence 3′ to intron 1 is necessary for inducible hepatic expression of the EPO gene.

In this study we have localized DNase I-hypersensitive sites to the immediate 3′-FS of the human EPO gene in liver nuclei isolated from tgEPO10 mice, which show liver-specific transgene expression. Digestion was seen at these sites even in the absence of added DNase I (see Fig. 1), presumably due to the action of endogenous nucleases. These sites were not hypersensitive to digestion in nuclei isolated from kidney or any other tissue studied. However, only a small proportion of kidney cells synthesize EPO even under conditions of severe anemia or hypoxia (17, 18), while tgEPO10 expression in liver involves a large proportion of the hepatocytes surrounding central veins (10). Thus, if the EPO gene 3′-FS were hypersensitive to DNase I digestion in the nuclei of EPO-expressing kidney cells, this sensitivity might not be detected.

The presence of DNase I-hypersensitive sites implies that factors present in liver nuclei bind to the 3′-FS of the human EPO gene. DNase I footprint analysis with a 3′-FS probe detected four regions of protection that span nt 3380–3505 (Fig. 3), mapping them to the same location as the DNase I-hypersensitive sites detected in liver chromatin (nt 3460–3560). Footprinting and gel shift studies suggested that both constitutive and inducible nuclear factors bound to oligonucleotides derived from the 3′-FS, and all of the factors were present in both liver and kidney nuclei. We propose that the 256-bp 3′-FS (the LIE; Fig. 3) is necessary and sufficient for regulated hepatic EPO gene expression, while in kidney other sequences (the kidney inducibility element, KIE) are also required. An alternative hypothesis, that the KIE functions independently of the LIE, seems less likely given the presence of LIE-binding factors in kidney.

Each oligonucleotide that bound nuclear factors contains repeated sequences (see Fig. 3): AGGGG(G) (in OL-1); CCTGGC (in OL-21); GCAGCAG and AGGTCC (in OL-23); and GACCT (in OL-33). The region of the AGGGG(G) repeats in OL-1 is necessary for factor binding. Both A residues can be mutated (OL-31) without loss of binding but the elimination of the last two G residues (as in OL-9) or first three G residues (as in OL-39 and -41) results in greatly decreased binding, suggesting that the GGGG(G) repeats are essential (see Fig. 5). A ribonucleoprotein has recently been shown (19) to bind to a 17-bp oligonucleotide, corresponding to nt −61 to −45 of the EPO gene 5′-FS, whose antisense strand can be aligned with the sense strand of OL-1 such that a region of 10/12 nt identity is seen. Further studies are needed to determine whether one factor binds to these related sequences. The binding site of OL-21 was localized to a 23-bp sequence at its 5′ end, which begins and ends with the direct repeat CCTGGC. Neither set of direct repeats in OL-23 alone is sufficient for binding, suggesting that sequences at both ends or in the central region are necessary for binding. The central region TGCAGCAGGTCCA is an imperfect palindrome that might be recognized by a dimeric transcription factor. OL-33 contains a direct repeat, YYYGACCT (Y = C or T), found in the binding site for liver nuclear factors Tf-LF1 and Tf-LF2 that is located in the 5′-FS of the transferrin and antithrombin III genes (20), suggesting that these factors may also bind to the EPO gene.

The functional significance of EPO gene sequences encompassing the nuclear-factor-binding sites was demonstrated by transient expression studies. The LIE functioned as a hypoxia-inducible enhancer in Hep3B cells, which inducibly express the endogenous EPO gene (4). Expression of the pSVcatEPO plasmids was induced 2.6- to 4.3-fold by hypoxia, compared with an average 9-fold induction of endogenous EPO production. Increased EPO levels are due in part to stabilization of EPO RNA during hypoxia (21), while CAT assays detect inducibility only at the level of transcription. Interactions between the 3′-FS enhancer and the EPO promoter may also play a role in transcriptional activation. The use of a heterologous promoter does allow us to attribute all positive effects observed to the 256-bp 3′-FS fragment, which, in hypoxic Hep3B cells, was a more powerful enhancer than the 236-bp SV40 sequence. When a 2.5-kb fragment of 3′-FS was tested (pSVcatEPO-2), the absolute level of expression in both 1% and 20% O_2 declined but the degree of inducibility was unchanged, suggesting that all positive cis-acting elements in the 3′-FS are included in the 256-bp fragment. The binding of at least four different nuclear factors within a sequence of only 122 bp (from 5′ end of OL-21 to 3′ end of OL-33; see Fig. 3) in a region far removed from the promoter is characteristic of enhancer elements (22). Binding sites for one or more of the factors described here may also be found in proximity to other hypoxia-inducible genes (23, 24).

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