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Regulation of transcription of the human erythropoietin receptor gene by proteins binding to GATA-1 and Sp1 motifs

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ABSTRACT

Erythropoietin (Epo), the primary regulator of the production of erythroid cells, acts by binding to a cell surface receptor (EpoR) on erythroid progenitors. We used deletion analysis and transfection assays with reporter gene constructs to examine the transcription control elements in the 5′ flanking region of the human EpoR gene. In erythroid cells most of the transcription activity was contained in a 150 bp promoter fragment with binding sites for transcription factors AP2, Sp1 and the erythroid-specific GATA-1. The 150 bp hEpoR promoter exhibited high and low activity in erythroid OCIM1 and K562 cells, respectively, reflecting the high and low levels of constitutive hEpoR expression. The GATA-1 and Sp1 binding sites in this promoter lacking a TATA sequence were necessary for a high level of transcription activation. Protein–DNA binding studies suggested that Sp1 and two other CCGCCC binding proteins from erythroid and non-erythroid cells could bind to the Sp1 binding motif. By increasing GATA-1 levels via co-transfection, we were able to transactivate the hEpoR promoter in K562 cells and non-erythroid cells, but not in the highly active OCIM1 cells, although GATA-1 mRNA levels were comparable in OCIM1 and K562. Interestingly, when we mutated the Sp1 site, resulting in a marked decrease in hEpoR promoter activity, we could restore transactivation by increasing GATA-1 levels in OCIM1 cells. These data suggest that while GATA-1 can transactivate the EpoR promoter, the level of hEpoR gene expression does not depend on GATA-1 alone. Rather, hEpoR transcription activity depends on coordination between Sp1 and GATA-1 with other cell-specific factors, including possibly other Sp1-like binding proteins, to provide high level, tissue-specific expression.

INTRODUCTION

Erythropoietin (Epo) is the hematopoietic cytokine responsible for proliferation and maturation of erythroid cells. Epo, which stimulates erythroid progenitors via binding to the cell surface erythropoietin receptor (EpoR), can function as a viability factor preventing apoptosis and as a differentiation factor stimulating heme production and globin synthesis. EpoR is a member of the superfamily of hematopoietic cytokine receptors, characterized by a single transmembrane domain and an extracellular domain with four common cysteines at the N-terminus and a Trp–Ser–X–Trp–Ser motif at the C-terminus which is required for activation (1). The intracellular domain contains no tyrosine kinase domain, although binding of Epo results in increased phosphorylation of EpoR itself and other cellular proteins. JAK2 and other specific non-receptor tyrosine kinases have been associated with the phosphorylation activity following Epo stimulation (2).

We and others have previously cloned the human erythropoietin receptor (hEpoR) gene encoding a 508 amino acid polypeptide in eight exons (3–5). Our analysis of a 15 kb genomic clone containing 2 kb of 5′ flanking sequence indicated that this 5′ region could provide a correctly initiated transcript when used as a DNA template with nuclear extracts from erythroid cells for *in vitro* transcription (3). The proximal 5′ region contains binding sites for transcription factors Sp1 at –17 bp and erythroid-specific GATA-1 at –45 bp and lacks a TATA sequence. The 15 kb cloned hEpoR gene was used to produce transgenic mice which expressed hEpoR in adult bone marrow and spleen, but not in heart, kidney, lung and liver (6).

To examine the transcription control elements within the 5′ flanking sequence of hEpoR we used deletion analysis and transfection assays. We used erythroid cell lines OCIM1 and K562, which have high and low levels of hEpoR on their cell surface respectively, as model systems for hEpoR transcription activation (7–10). We describe a minimal hEpoR promoter that contains binding sites for transcription factors Sp1 and GATA-1

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and that reflects the high and low levels of constitutive hEpoR transcription activity in transient transfection assays. Protein binding studies provide evidence of multiple proteins binding to the Sp1 and GATA-1 binding motifs. Data are presented to suggest that coordination of Sp1 and GATA-1 with other cellular factors act to provide a high level of hEpoR transcription activation.

MATERIALS AND METHODS

Cell culture

OCIM1 cells (11) were maintained in alpha minimal essential medium containing 10% fetal bovine serum (FBS). K562 cells (12) were maintained in RPMI 1640 medium supplemented with 10% FBS. HeLa cells were cultured in EMEM medium supplemented with 10% FBS. The cells were allowed to expand in an atmosphere of 5% CO_2.

Plasmid construction

Plasmids for transfection assays of promoter strength were constructed using the *Xho*I and *Hin*dIII restriction enzyme sites within the PGL2Basic luciferase plasmid (Promega, Madison, WI). Insert DNA was constructed by PCR using sequence-specific oligonucleotides flanked by *Hin*dIII and *Xho*I restriction enzyme sites. Fidelity of the constructs was verified by restriction enzyme mapping and sequencing of the junction region. At least two constructs subcloned for each deletion mutant consisting of ≥445 bp were tested. Two mutations of the GATA-1 binding motif were constructed using the luciferase reporter plasmid Δ194 (containing the human erythropoietin receptor promoter extending to –194 bp 5′) in which AGATAA was replaced with CTGCAG (*Pst*I restriction site) for ΔGATA-a and with CTCGAG (*Xho*I restriction site) for ΔGATA-b. A mutation of the Sp1 binding motif was constructed in which the Sp1 binding motif was replaced with GGATCC (*Bam*HI restriction site). Plasmid pSVGH (SV40 promoter linked to a growth hormone reporter gene) was used to evaluate transfection efficiency. PGL2 Control (Promega, Madison, WI) contained the simian virus (SV40) promoter and enhancer sequence linked to the luciferase reporter gene, while the PGL2 Basic (Promega) plasmid contained no promoter or enhancer sequences. The eukaryotic expression vector for human GATA-1 contained a Rous sarcoma virus (RSV) promoter linked to the human GATA-1 cDNA. An $^{A}\gamma$-globin reporter gene construct consisted of an $^{A}\gamma$-globin promoter fragment extending to –160 bp 5′ of the cap site linked to a luciferase reporter gene.

Transient transfection analysis

Transfection of plasmid DNA was carried out by electroporation (Gene Pulser; BioRad Laboratories, Hercules, CA) as previously described (13). The constructs were co-transfected with plasmid pSVGH as an internal control. Cells were cultured for 48 h in appropriate media. Luciferase assays were performed by direct lysis of cells in lysing buffer containing acetylCoA (Promega, Madison, WI) and assay of the supernatant for luciferase activity using a Luminometer (Analytical Luminescence Laboratory, San Diego, CA). Growth hormone was quantitated by radioimmunoassay using ^{125}I-labeled anti-growth hormone antibody (Nichols Institute, San Juan Capistrano, CA).

Northern blot analysis

Cellular mRNA was prepared using an oligo(dT) column (Pharmacia). For Northern blot analysis 2 μg/lane mRNA were loaded on a 1% agarose gel containing 2.2 M formaldehyde. The mRNAs blotted onto Nytran (Schleicher & Schuell Inc., Keene, NH) filters were hybridized with a random primed ^{32}P-labeled cDNA probe for GATA-1 and β-actin. Hybridization was carried out in 50% formamide, 10% dextran sulfate, 1% SDS at 42°C.

Gel mobility shift

The gel mobility shift assay was carried out as previously described (14). Nuclear extracts from OCIM1 and HeLa continuous cell lines were prepared following the method of Dignam *et al.* (15). Double-stranded synthetic DNA fragments were used as probes and DNA competitors. Oligonucleotides were synthesized on an Applied Biosystems 308B DNA synthesizer (Foster City, CA). Complementary strands were annealed at 65°C for 5 min and the resultant double-stranded DNA fragments were gel purified. The DNA probes were ^{32}P-labeled at the 5′-ends using T4 polynucleotide kinase. The binding reaction was carried out in 15 μl containing 0–10 μg OCIM1 or HeLa cell nuclear extract, 1 μg poly(dI·dC) and specific competitor DNA as indicated. The binding buffer was the same as that used for DNase I footprinting assays (see below) and consisted of 10 mM HEPES, pH 7.6, 48 mM KCl, 0.04 mM EDTA, 8 mM $MgCl_2$ with protease inhibitors.

DNase I footprinting

In vitro DNase I footprinting was performed according to the method of Briggs *et al.* (16) with the following modification. The DNA probes were constructed using PCR-amplified DNA to generate a DNA fragment extending from –250 to –24 bp 5′ of the cap site for the human erythropoietin receptor gene and another probe extending from –136 bp 5′ to +66 bp 3′ of the cap site. Labeling of the sense or antisense strand of the probe was achieved by using the appropriately ^{32}P-labeled primer. Aliquots of probe (20 000 c.p.m.) and nuclear extract (50–100 μg) were incubated for 30 min at 30°C in the binding buffer described above for gel mobility shift assay with 2 μg poly (dI·dC). DNase I (1 U) was added on ice and incubated on ice for 1 min. DNase I activity was stopped by addition of 1 vol 50 mM EDTA with 0.2% SDS, 100 μg/ml tRNA and 500 μg/ml proteinase K and incubated at 55°C for 1 h. After phenol/chloroform extraction and ethanol precipitation the sample was resuspended in loading buffer containing 90% formamide and analyzed on a 6% acrylamide–7 M urea sequencing gel.

RESULTS

Functional analysis of the EpoR 5′ flanking region

As model systems we used erythroid cell lines OCIM1 and K562 and non-erythroid HeLa cells. OCIM1 cells express high levels of hEpoR, with 3000 Epo binding sites on the cell surface (7). In K562 cells the hEpoR gene is active, but only at low levels, with only a few Epo binding sites on the cell surface (8,9). The level of hEpoR mRNA transcripts in OCIM1 cells (4.8×10^{-3} ng/μg mRNA) is at least one order of magnitude higher than the level of hEpoR mRNA transcripts in K562 cells (2.3×10^{-4} ng/μg mRNA) (10), suggesting that the regulation of hEpoR expression

is reflected, in part, at the level of transcription in OCIM1 and K562 cells.

The 5′ flanking region of the hEpoR gene was analyzed using a luciferase reporter gene and transient transfection assays to identify elements contributing to specific hEpoR gene expression. A series of deletion mutation constructs, Δ1778, Δ937, Δ445, Δ150 and Δ+2, was made extending 5′ to –1778, –937, –445 and –150 bp upstream and +2 bp downstream from the transcription start site, respectively. The longest construct (Δ1778) includes five CACCC motifs located at –451, –1571 and –1772 bp and at –465 and –799 bp in the antisense direction 5′ of the transcription start site, with an additional CACCC motif in the antisense direction at +84 bp in the transcribed untranslated region of the hEpoR gene. The 5′ flanking region of the hEpoR gene also contains extensive homology to Alu repeat sequences and a purine-rich region of 123 bp consisting only of Gs and As extending from –322 to –444 bp. The 5′ CACCC motifs and purine-rich region were deleted in the Δ150 construct, which retains the binding motifs for GATA-1 and Sp1 transcription factors. All transient transfection assays with these hEpoR deletion constructs were carried out with co-transfection of pSVGH and the activity of growth hormone was quantitated and used to determine transfection efficiency.

The hEpoR reporter gene constructs were most active in OCIM1 cells with a high level of endogenous hEpoR activity, compared with K562 and non-erythroid HeLa cells (Fig. 1), reflecting the lower or absent activity of the endogenous hEpoR gene in K562 cells and HeLa cells, respectively. The relative transcription activities of the Δ1778, Δ937, Δ445 and Δ150 constructs suggested regions of positive and negative regulation. The Δ937 construct fell to 75% ($P = 0.028$) of the activity of the Δ1778 construct in OCIM1 cells, suggesting a potential region of positive regulation; the regions between –937 and –445 bp and between –445 and 150 bp exhibited some negative regulation and the activity of Δ150 was 2-fold greater than the activity of Δ937 ($P = 0.002$). Δ150 was the hEpoR construct with the greatest level of activity. The Δ+2 construct without any 5′ flanking sequence exhibited no transcription activation. These constructs exhibited a similar relative behavior in K562 cells, but with a lower level of activity. These data suggest that the 150 bp 5′ of the transcription start site can function as a proximal promoter for transcription activation of the hEpoR gene in erythroid OCIM1 and K562 cells and may be erythroid specific.

In non-erythroid HeLa cells the relative activity of the hEpoR constructs did not follow the behavior of that observed for erythroid OCIM1 or K562 cells and appeared to depend on the length of the 5′ flanking region (Fig. 1). The greatest activity was observed with the longest construct, Δ1778. The activities observed for Δ937 and Δ445 were half that obtained for Δ1778 ($P = 0.005$ and $P = 0.009$, respectively) and the activity of Δ150 was ~5-fold lower than that obtained for Δ1778 ($P < 0.001$). As expected, transcription activity decreased to baseline levels for the Δ+2 construct, which lacks 5′ flanking sequence.

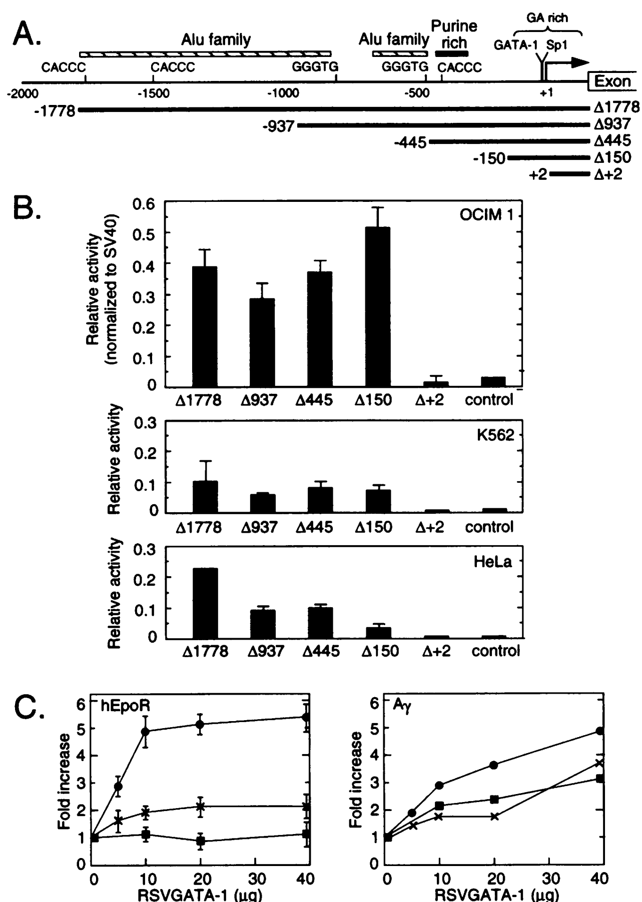

Figure 1. Transient transfection assay of the hEpoR 5′ region. (**A**) Human EpoR gene fragments extend 5′ to –1778 (Δ1778), –937 (Δ937), –445 p (Δ445), –150 (Δ150) and +2 bp (Δ+2) relative to the transcription start site were used in luciferase reporter gene constructs. (**B**) The hEpoR reporter gene constructs were assayed in the erythroid OCIM1 (top panel) and K562 (middle panel) cell lines and the non-erythroid HeLa cell line (lower panel). Data normalized to SV40 promoter activity are plotted. The control construct contains no promoter. (Mean SV40 promoter activities were 5041, 20 607 and 25 000 relative luciferase units in OCIM1, K562 and HeLa cells, respectively.) (**C**) Increasing amounts of RSV-GATA-1 expression plasmid were co-transfected with the hEpoR Δ150 or human $^{A}\gamma$-globin reporter gene constructs. Data are normalized to activity in the absence of RSV-GATA-1. Constructs were assayed in OCIM1 (squares), K562 (circles) and HeLa (×) cells. (The relative activities of the $^{A}\gamma$-globin promoter in OCIM1, K562 and HeLa cells without RSV-GATA-1 were 0.8, 0.1 and 0.1 of the SV40 promoter, respectively.)

Transactivation of the hEpoR promoter by GATA-1

The prominent feature of the Δ150 construct is the presence of binding motifs for transcription factors GATA-1 and Sp1. GATA-1 exhibits erythroid specificity and is associated with transcription activation of several erythroid-specific genes, including globin (17). To determine the ability of GATA-1 to transactivate the EpoR promoter we co-transfected an expression vector for the human GATA-1 protein (RSV-GATA-1) with the hEpoR reporter gene constructs (Fig. 1C). An expression plasmid containing RSV-CAT was used to maintain a constant level of DNA in these co-transfection experiments. In erythroid K562 cells increasing GATA-1 resulted in transcription activation of the hEpoR promoter up to 5-fold. Transactivation appeared to saturate as the amount of RSV-GATA-1 increased beyond 10 μg/reaction. Increasing GATA-1 levels in non-erythroid HeLa cells resulted in transactivation of the hEpoR promoter by 2-fold or greater. Surprisingly, in erythroid OCIM1 cells with high levels of constitutive hEpoR expression (7,10) and in which the hEpoR

promoter exhibits high activity, increasing GATA-1 levels had no effect on hEpoR promoter activity. Co-transfection with the Δ1778 hEpoR construct with RSV-GATA-1 also produced no increase in transcription activity in OCIM1 cells, but increased transcription activity in K562 and HeLa cells, comparable with results obtained with Δ150 (data not shown). These data suggest that when the activity of the hEpoR promoter is low, increasing GATA-1 expression is able to transactivate the hEpoR promoter and that transactivation by GATA-1 appears to saturate. Although an Sp1 binding motif is also contained within the hEpoR proximal promoter, co-transfection with an Sp1 expression vector did not further increase hEpoR activity in either K562 or OCIM1 cells.

To determine if the transactivation behavior of GATA-1 for the hEpoR promoter in K562 and OCIM1 cells was promoter specific we repeated the experiment with another erythroid-specific promoter, a minimal $^{A}\gamma$-globin promoter extending to –160 bp 5′ of the cap site, which also contains a GATA-1 binding site. Co-transfection with increasing amounts of RSV-GATA-1 was able to increase $^{A}\gamma$-globin promoter activity in both K562 and OCIM1 cells by up to 5- and 2-fold, respectively (Fig. 1C). The inability of GATA-1 to transactivate the hEpoR promoter in OCIM1 cells appears to be specific for hEpoR. Northern blot analysis of GATA-1 mRNA in OCIM1 and K562 cells indicated that the ratio of GATA-1 mRNA in K562 cells to OCIM1 cells was 1.09 (data not shown) and that the RSV-GATA-1 expression vector was active in both K562 and OCIM1 cells. These data suggest that the differences observed in hEpoR promoter activity between K562 and OCIM1 cells cannot be attributed to differences in GATA-1 levels alone.

Protein binding to the hEpoR promoter

To identify possible control elements within the hEpoR promoter, we determined regions of DNA which could bind to nuclear proteins. For DNase I footprinting we used a ^{32}P-labeled DNA probe beginning at –236 bp 5′ of the cap site extending 3′ and nuclear extracts from erythroid OCIM1 cells (Fig. 2). A region around –175 was protected from DNase I digestion, as well as a region containing an AP2 site located at –101. Sequence analysis of the –175 region did not reveal any homology with other known protein binding consensus sequences. A second probe beginning at –136 bp 5′ of the cap site and labeled with ^{32}P was used to examine the binding of nuclear proteins in the region around the Sp1 and GATA-1 binding sites. Incubation of probe with nuclear extracts from OCIM1 cells followed by treatment with DNase I revealed a region of protection around the GATA-1 binding site. The DNase I footprint generated with OCIM1 nuclear extracts also showed protection in the region containing the Sp1 binding site. These data suggest that the footprinted region around –175 bp and the region containing the AP2 site at –101 in addition to the GATA-1 and Sp1 sites may function as protein binding regions *in vivo* and are possible candidates for transcription regulatory regions.

Protein binding to Sp1 and GATA-1 binding motifs

To characterize further the binding of nuclear proteins to the Sp1 and GATA-1 binding motifs we used gel mobility shift assays with a DNA probe extending from –63 bp 5′ to +1 bp 3′ of the cap site. When the ^{32}P-labeled probe was incubated with OCIM1 nuclear extract and analyzed by gel electrophoresis we identified three major bands (1, 2 and 4) and a band of lesser intensity (band 3) corresponding to various DNA–protein complexes (Fig. 3). The presence of the faint band between bands 3 and 4 was not consistent and appeared to be non-specific. Competition with cold probe (ER) was able to effectively displace bands 1–4 (Fig. 3A, lanes 1–4), suggesting that these bands represented specific binding of OCIM1 nuclear proteins to the hEpoR promoter. Bands representing binding to the Sp1 consensus sequence were identified using an Sp1 (SP1) DNA competitor unrelated to hEpoR (Fig. 3A, lanes 5–9). This competitor markedly reduced the intensities of band 1, 3 and 4. A 20 bp Sp1 competitor from hEpoR (SP1ER) also reduced the intensity of bands 1, 3 and 4 (Fig. 4). Antibodies specific to Sp1 protein (anti-SP1; Santa Cruz Biotechnology Inc., Santa Cruz, CA) incubated with the reaction mixture resulted in a decrease in the intensity of band 1 and the appearance of a new band of high molecular weight (representing a new complex consisting of antibody, Sp1 protein and probe) (Fig. 3A). These data suggest that band 2 represents protein binding to the GATA-1 binding motif, that band 1 represents

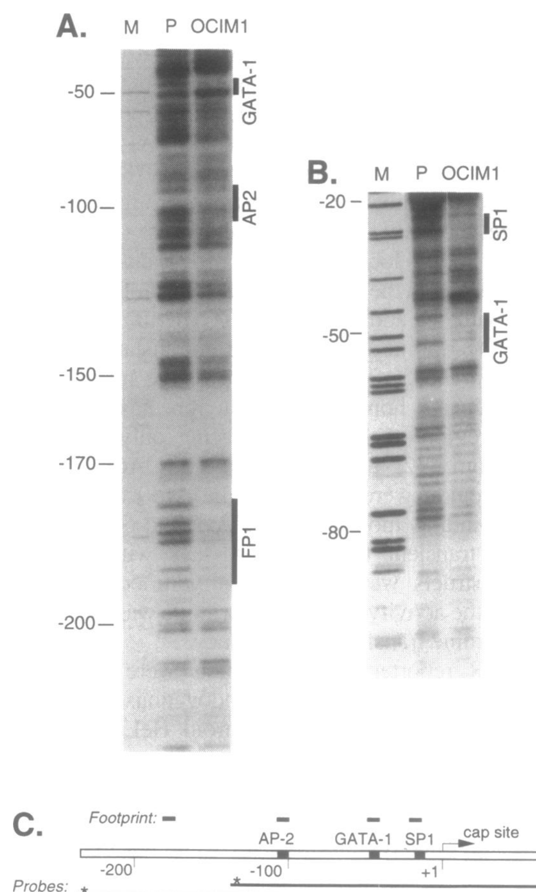

Figure 2. *In vitro* footprinting of the hEpoR proximal promoter. Probes from the hEpoR proximal promoter extending from –250 to –24 bp (**A**) and from –136 to +66 bp (**B**) relative to the cap site were ^{32}P-labeled at the 5′-end (**C**). Probes were incubated with OCIM1 (OCIM-1) nuclear extract followed by DNase I digestion. P indicates probe alone; M indicates the M13 sequencing marker. Regions containing binding sites for GATA-1 (GATA-1), AP2 (AP2) and Sp1 (SP1) and a region around –175 bp (FP1) were footprinted.

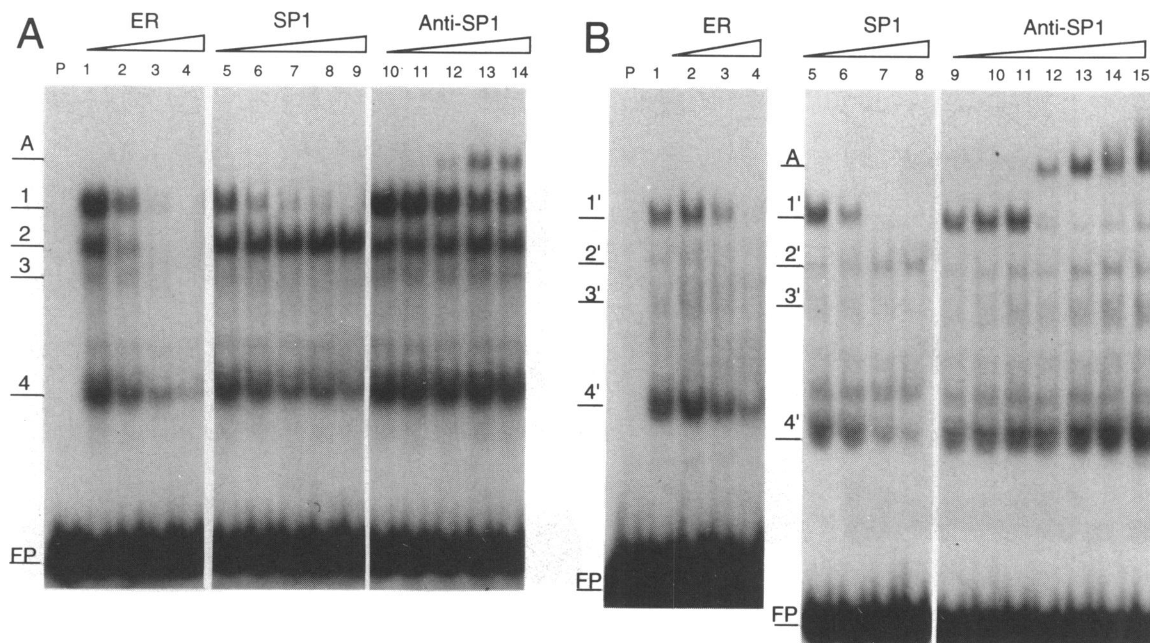

Figure 3. Gel mobility shift assay of the hEpoR promoter and Sp1 antibody. (**A**) A double-stranded hEpoR fragment extending from the cap site to –63 bp 5′ was ^{32}P-labeled and incubated with OCIM1 nuclear extract or no nuclear extract (P) as indicated. Lane 1 contains no specific DNA competitor. Lanes 2–4 contain cold probe (10, 30 and 100×). Lanes 5–9 contain Sp1-specific DNA competitor (10, 25, 50, 75 and 100×). Lanes 10–14 contain anti-Sp1 antibody (10, 25, 50, 80 and 100 ng). (**B**) Probe was incubated with HeLa nuclear extract or no nuclear extract (P) as indicated. Lane 1 contains no specific DNA competitor. Lanes 2–4 contain cold probe (10, 30 and 100×). Lanes 5–8 contain Sp1-specific DNA competitor (1, 10, 100 and 500×). Lanes 9–15 contain anti-Sp1 antibody (1, 10, 100 and 300 ng and 1 and 3 μg).

binding of Sp1 protein and that bands 3 and 4 represent other non-Sp1 nuclear proteins binding to the CCGCCC Sp1 binding motif.

Incubation of probe with HeLa nuclear extract resulted in a gel mobility shift pattern consisting of two major bands (1′ and a broad band, 4′) and two bands (2′ and 3′) of lesser intensity (Fig. 3B) which could be competed with ER. Bands 1′, 3′ and 4′ from HeLa nuclear extract behaved similarly to bands 1, 3 and 4 from OCIM1 nuclear extract when incubated with SP1 and the Sp1-specific antibody anti-SP1. These data suggest that, as with OCIM1 nuclear extracts, band 1′ represents binding of Sp1 protein and bands 3′ and 4′ represent other non-Sp1 nuclear protein binding to the Sp1 binding motif.

For OCIM1 nuclear extract a GATA-1 DNA competitor from the ε-globin gene promoter (GATA-1ε) was able to reduce the intensity of band 2 (Fig. 4A, lanes 1–3). A synthetic 20 bp DNA fragment centered at the hEpoR GATA-1 binding motif (GATA-1ER) was also able to compete for binding of protein represented in band 2 (Fig. 4A, lanes 4–9). These data suggest that the DNA–protein complex represented by band 2 corresponds to protein binding to the GATA-1 binding motif. Incubation with anti-GATA-1 antibody (N6) specific for GATA-1 protein (Santa Cruz Biotechnology Inc., Santa Cruz, CA) (18) resulted in a decrease in the relative intensity of band 2 with a marked increase in intensity of bands 1, 3 and 4 with increasing amounts of anti-GATA-1 antibody (Fig. 4B), similar to the competition pattern observed with the GATA-1ER competitor (Fig. 4A). Note that no high molecular weight band appeared, indicating the lack of formation of an antibody–GATA-1 protein–DNA probe complex. Incubation with antibodies specific for TFIID (anti-TFIID; Santa Cruz Biotechnology Inc., Santa Cruz, CA) or with a synthetic DNA fragment containing a TFIID binding motif had no effect on the gel mobility shift pattern (Fig. 4C).

HeLa cells, although they do not express GATA-1 protein, do express GATA-2 protein, which has a DNA binding motif similar to GATA-1 protein (19,20). The intensity of band 2′ is low (Fig. 4D). However, incubation with the specific DNA competitor GATA-1ER, which contains the GATA-1 binding motif, further decreases the intensity of band 2′, with a marked increase in the intensity of bands 1′ and 3′, a pattern similar to that obtained with OCIM1 nuclear extracts. These data suggest that band 2′ may represent binding of protein from HeLa nuclear extract, possibly GATA-2, to the GATA-1 motif, but at a much lower level than that observed for OCIM1 nuclear extract.

Functional studies of the hEpoR promoter

Truncated deletions were constructed around the DNase I footprinted regions of the hEpoR promoter beginning 5′ from –194 (Δ194), –116 (Δ116), –68 (Δ68), –29 (Δ29) and +3 bp (Δ+3) relative to the cap site and extending 3′ to +123 bp (Fig. 5A). As expected, the Δ194, Δ116 and Δ68 constructs were at least 6-fold more active in OCIM1 cells than in HeLa cells (Fig. 5). Co-transfection with the GATA-1 expression vector in HeLa cells also resulted in transactivation of the promoter activity of Δ194, Δ116 and Δ68 (data not shown). In OCIM1 cells the region between –194 and –116 bp containing the DNase footprinted region around –175 bp provided some negative regulatory activity, as deletion of this region resulted in a ≥ 2-fold increase ($P = 0.002$) in transcription activity (consistent with the data in

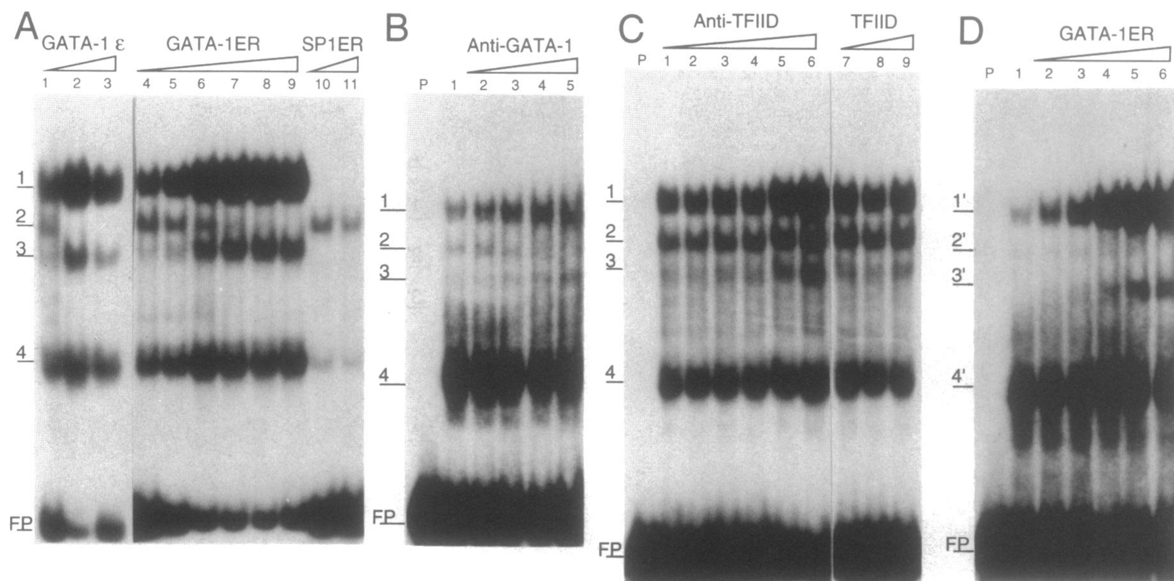

Figure 4. Gel mobility shift assay of the hEpoR promoter region. (**A**) The hEpoR promoter probe was incubated with OCIM1 nuclear extract. Lanes 1 and 4 contain no specific DNA competitor. Lanes 2 and 3 contain GATA-1 DNA competitor from the ε-globin gene sequence (165 and 400×). Lanes 5–9 contain a 20 bp GATA-1 DNA competitor from hEpoR (20, 40, 60, 80 and 100×). Lanes 10 and 11 contain a 20 bp Sp1 DNA competitor from hEpoR (10 and 20×). (**B**) The hEpoR probe was incubated with OCIM1 nuclear extract. Lanes 1–5 contain anti-GATA-1 antibody (1, 10, 100 and 300 ng and 1 μg). (**C**) The hEpoR probe was incubated with OCIM1 nuclear extract. Lanes 2–6 contain anti-TFIID antibody (10, 100 and 300 ng and 1 and 3 μg). Lanes 7–9 contain TFIID-specific DNA competitor (1, 10 and 100×). (**D**) The hEpoR probe was incubated with HeLa nuclear extract. Lane 1 contains no specific DNA competitor. Lanes 2–6 contain a 20 bp GATA-1-specific DNA competitor from hEpoR (20, 40, 60, 80 and 100×).

Fig. 1B). The region between –68 and –116 bp containing the AP2 site provided positive regulatory activity and deletion of this region resulted in a decrease in the transcription activity of Δ68 compared with Δ116 ($P = 0.001$).

The Δ29 hEpoR construct represents deletion of the GATA-1 binding motif and 5′ sequences. We have also constructed two mutations of the GATA-1 binding motif within the Δ194 construct using site-directed mutagenesis to replace the GATA-1 binding motif with CTGCAG for ΔGATA-a and CTCGAG for ΔGATA-b. When assayed in OCIM1 cells deletion or mutation of the GATA-1 binding site at –45 bp reduced transcription activity to 50–70% of the native construct (Figs 5 and 6). In contrast, deletion or mutation of the GATA-1 binding site in HeLa cells did not decrease transcription activity. While increasing GATA-1 levels in OCIM1 cells was not able to further increase transcription activity (Fig. 1C), the reduction in transcription activity observed by mutation or deletion of the GATA-1 binding site demonstrates its importance in transcription activity in OCIM1 cells. Furthermore, the effect of mutation or deletion of the GATA-1 binding site on transcription activity in HeLa cells suggests that although GATA-2 and GATA-1 share similar DNA binding sites, GATA-2 in HeLa cells does not act to increase hEpoR promoter activity.

To determine the dependence of transcription activation on the Sp1 binding site, we used site-directed mutagenesis to alter the Sp1 binding motif in the Δ194 construct, resulting in plasmid ΔSp1. When transfected into OCIM1 cells promoter activity was markedly reduced (Fig. 6). The double mutation of both Sp1 and GATA-1 binding sites in plasmid ΔSp1ΔGATA1 further reduced promoter activity in OCIM1 cells to that observed for the control construct containing no promoter. Promoter activities of ΔSp1 and ΔSp1ΔGATA1 were also decreased compared with the unmutated hEpoR promoter when these constructs were transfected into HeLa cells.

Although mutation of the Sp1 site (ΔSp1) resulted in low transcription activity of the hEpoR promoter, this construct was still responsive to transactivation by GATA-1. Co-transfection of ΔSp1 with the GATA-1 expression vector resulted in a 6-fold increase in transcription activity in HeLa cells (Fig. 6). Surprisingly, transactivation by GATA-1 was also observed when ΔSp1 was co-transfected into OCIM1 cells. These data suggest that transactivation by GATA-1, not observed when hEpoR promoter activity is high, can be restored when promoter activity is markedly reduced and that a high level of hEpoR transcription activation depends on coordination of both Sp1 (or Sp1-like) and GATA-1 transcription factors with other cell-specific factors.

DISCUSSION

We have previously shown that a genomic fragment containing 700 bp of 5′ sequence flanking the hEpoR coding region was functional in an *in vitro* transcription system based on nuclear extracts from human erythroid cells and provided a RNA transcript correctly initiated at 134 bp 5′ of the translation start site (3). Recently we reported that a 15 kb hEpoR transgene with 2 kb of 5′ flanking sequence was able to direct hematopoiesis-specific expression (with low level brain expression) in transgenic mice (6). Here we provide an analysis of the 5′ region of the hEpoR gene with emphasis on the proximal promoter. All the constructs containing the hEpoR 5′ flanking region extending from –150 bp or more 5′ of the transcription start site, which contain the binding sites for transcription factors Sp1 and the erythroid-specific

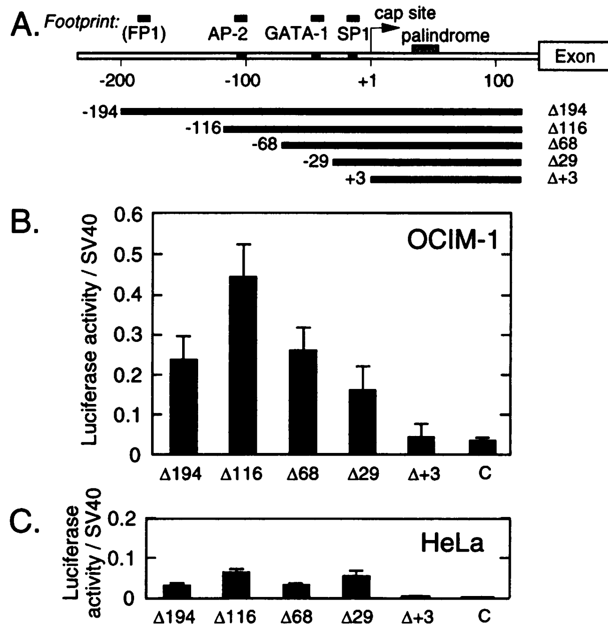

Figure 5. Transfection assay of the hEpoR promoter. (**A**) Human EpoR reporter gene deletion mutants were constructed extending to –194 (Δ194), –116 (Δ116), –68 (Δ68), –29 (Δ29) and +3 bp (Δ+3) relative to the cap site. (**B**) The hEpoR constructs were transfected into OCIM1 cells. Data normalized to SV40 promoter luciferase activity are plotted. The control construct (C) contains no promoter. (**C**) Data obtained for the hEpoR constructs assayed in HeLa cells are plotted.

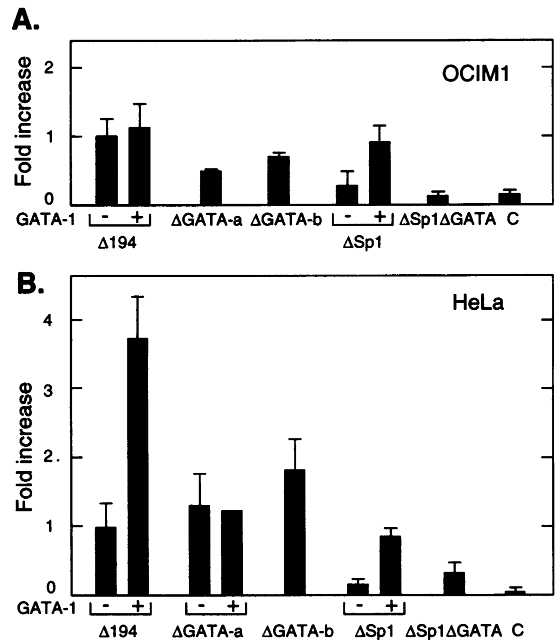

Figure 6. Transfection assay with mutated hEpoR promoters. The hEpoR construct Δ194 was mutated at the GATA-1 binding site (ΔGATA-a and ΔGATA-b), at the Sp1 binding site (ΔSP1) and at both the GATA-1 and Sp1 binding sites (ΔSP1ΔGATA). The control construct (C) contains no promoter. (**A**) Constructs were assayed in OCIM1 cells and data normalized to Δ194 activity. Also indicated are co-transfections with RSV-GATA-1 (+). (**B**) Constructs were assayed in HeLa cells and data normalized to Δ194 (Native) activity.

GATA-1, were transcriptionally active in erythroid OCIM1 and K562 cells (Fig. 1).

The CACCC motif, which has been associated with transcription activity of globin and other erythroid promoters, is usually located within or in close proximity to the promoter (20,21). For the hEpoR gene five CACCC motifs are located in more distal 5′ regions (3) and deletion of these motifs had a <2-fold effect on promoter activity. The murine EpoR gene contains three CACCC motifs localized within 400 bp 5′ of the transcription start site (22), with one at –261 bp which contributes significantly to murine EpoR promoter activity (23). The region between –1778 and –937 bp in the hEpoR gene includes homology to Alu repetitive sequences and is associated with some positive control. The murine EpoR gene contains a rodent-specific repetitive sequence between –1703 and –1063 bp with negative regulatory activity (24). Additional regulatory motifs found in the hEpoR promoter include a region with an AP2 binding site located at –101 bp in the hEpoR promoter (Fig.2) with some positive regulatory activity (Fig. 5) and the region between –116 and –194 bp containing a DNase I footprinted region (Fig. 2) associated with negative regulatory activity (Fig. 5). These differences between the human and murine regulatory elements illustrate that the human and murine EpoR genes are not under identical regulatory control.

In erythroid cells much of the hEpoR transcription activity is contained within the 150 bp fragment 5′ of the cap site with activity equal to or greater than the longest Δ1778 construct (Fig. 1). Furthermore, the relative activity of the hEpoR constructs mirrored the high and low levels of constitutive hEpoR expression in OCIM1 and K562 cells, respectively. In non-erythroid HeLa cells the hEpoR promoter is dependent on the length of the 5′ flanking region and the activity of Δ150 is >5-fold lower than the activity of Δ1778. Mutation or deletion of the GATA-1 site markedly reduced transcription activity in OCIM1 cells, but exhibited no reduction of transcription activity in HeLa cells (Fig. 6). In the human EpoR gene only one perfect GATA-1 binding consensus sequence at –45 bp is found in the 5′ flanking region (3). The variation in GATA-1 binding motifs (19,20) suggests that other GATA-1 binding sites might be found in the hEpoR gene. It is possible that other GATA-1 binding sites outside the proximal promoter can further contribute to the tissue-specific expression of hEpoR (25). For example, for the murine EpoR it has been suggested that a GATA-1 site within IVS1 of murine EpoR provides additional positive regulatory activity (26).

Increasing GATA-1 levels results in transactivation of hEpoR promoter activity in erythroid K562 and non-erythroid HeLa cells (Fig. 1C). GATA-1, associated predominantly with transcription activation of erythroid-specific genes, is found in other cell lineages, such as megakaryocytes and mast cells (17). EpoR transcripts have also been observed in non-erythroid hematopoietic cells expressing GATA-1 (27,28), consistent with the importance of GATA-1 in activation of the endogenous EpoR gene. The low level of hEpoR promoter activity in HeLa cells (Fig. 1),which do not express GATA-1, is similar to the inactivity of the murine EpoR promoter in mouse fibroblasts and T and B cells (22). Although previous reports have shown that GATA-1 is able to transactivate the murine EpoR promoter in erythroid (29) and non-erythroid cells (30), we found that increasing the level of

GATA-1 alone was not sufficient to further increase the activity of the hEpoR promoter in erythroid OCIM1 cells (Fig. 1C). These data suggest that transactivation of hEpoR by GATA-1 may be saturable, as observed with K562 and HeLa cells at high levels of co-transfected RSV-GATA-1. However, the high level of hEpoR activity does not appear to depend on the level of GATA-1 alone, as Northern blot analysis indicates that GATA-1 mRNA in OCIM1 and K562 cells are comparable (data not shown). Furthermore, phosphorylation studies of GATA-1 suggest that DNA binding and transcription activity are not affected by variation in phosphorylation of GATA-1 protein (31). However, other differences in translational or post-translational processing or differences in conformation or structure of GATA-1 protein which affect the activity of GATA-1 protein are possible. The saturation of GATA-1 transactivation in OCIM1 cells appears to be specific for hEpoR, as increasing GATA-1 levels were able to transactivate a $^{A}\gamma$-globin promoter construct in co-transfection assays using OCIM1 cells.

The high and low levels of hEpoR activity in erythroid cells are particularly relevant to differential expression of the hEpoR gene during erythropoiesis. For human primary erythroid cells BFU-E (burst forming units–erythroid) exhibit <200 hEpoR/cell (32). Stimulation by Epo results in differentiation and maturation to CFU-E (colony forming unit–erythroid) which contain 1100 hEpoR/cell as the erythroid precursors become Epo-dependent. With increasing maturation the number of receptors decreases and none are detected on human reticulocytes. Epo stimulation of erythroid progenitors and subsequent proliferation and differentiation is accompanied by an increase in GATA-1 levels (33). Transactivation of the EpoR promoter by GATA-1 and the association between increased EpoR expression and increased GATA-1 levels in primary erythroid cultures suggest that GATA-1 contributes significantly to activation of the EpoR gene. The data presented here also indicate that expression of GATA-1 alone is insufficient to determine the level of activity of the EpoR gene.

Adjacent to the GATA-1 binding motif at –45 bp is the Sp1 binding motif at –17 bp, but no TATA sequence. As with many TATA-less promoters, hEpoR contains an initiator sequence with the Sp1 binding site in proximity, which maps to a stretch of 27 nt with 89% homology to the murine EpoR gene (3) and overlaps with a 17 bp motif with 76% homology to the initiator sequence region for the IL-3β receptor gene (34). For transcription initiation TBP (TATA binding protein) is still required as part of the transcription complex and Sp1 in conjunction with other co-activators and a multisubunit TFIID complex are able to direct transcription (35). However, for the hEpoR promoter we observed no direct interactions between the promoter and TFIID in gel mobility shift assays using TFIID-specific antibodies and a TFIID-specific DNA competitor (Fig. 4C). Using antibodies specific for Sp1 and Sp1-specific DNA competitors we observed explicit binding of Sp1 and two non-Sp1 proteins to the hEpoR promoter (Fig. 3). Sp1 was a critical element in transcription activation of the hEpoR promoter, as mutation of the Sp1 binding motif (ΔSp1) resulted in a marked decrease in hEpoR transcription activity (Fig. 6). However, differential activation of the hEpoR promoter in erythroid K562 and OCIM1 cells was not due to limiting amounts of Sp1, as co-transfection with the hEpoR promoter and an Sp1 expression vector did not affect hEpoR promoter activity (data not shown). Little or no high molecular weight material corresponding to simultaneous binding of GATA-1 and Sp1 was detected. Interestingly, in gel shift assays with probe containing the Sp1 and GATA-1 binding motifs competition for nuclear protein binding to the GATA-1 motif using GATA-1 DNA competitors or anti-GATA-1 antibody resulted in apparent increased protein binding to the Sp1 motif (and vice versa to a lesser extent), suggesting a relationship (possibly interference) between binding to these two motifs, likely due to their close proximity. In fact, increasing the spacing between these motifs changes promoter activity (data not shown).

When the GATA-1 expression vector was co-transfected with the ΔSp1 plasmid with low activity into OCIM1 cells we observed transactivation by increased GATA-1. These data suggest that both Sp1 and GATA-1 are critical elements in determining transcription activation of the hEpoR promoter and that coordination of Sp1 and GATA-1 with other cell-specific elements, possibly including other Sp1-like nuclear proteins, determines the high level of hEpoR transcription activation. Interaction between transcription factors Sp1 and GATA-1 has been hypothesized as a possible mechanism for stage-specific expression of globin genes (36) and for erythroid-specific expression of non-globin genes (21). In addition, the sensitivity to the hEpoR promoter to alteration of the GATA-1 binding sequence suggests that expression of EpoR in non-erythroid tissues, such as endothelial cells (10) and brain (6,37,38), may represent coordinate expression of Sp1 with other GATA-like proteins (19,20). Indeed, our results suggest that in non-erythroid cells GATA-2 may interact with this motif and may confer little, or even negative, transcription control.

The relative activities of the hEpoR reporter gene constructs in erythroid OCIM1 and K562 cells reflected the high and low levels of endogenous gene expression in these cells, suggesting that soluble factors may be sufficient to account for the differences in hEpoR gene expression in erythroid cells. In contrast, although the Δ1778 hEpoR construct was active in HeLa cells (Fig. 1), the endogenous hEpoR gene is silent. Furthermore, an hEpoR transgene containing this 5′ flanking region exhibits appropriate tissue-specific expression in transgenic mice (6). These data suggest that erythroid-specific expression of hEpoR may be dependent on other factors not present in transient transfections, such as DNA structure (chromatin) or DNA modification (methlyation), to provide negative regulation or suppression of hEpoR expression in non-erythroid cells or may require additional regulatory sequences 3′ of the transcription start site which are included in the hEpoR transgene. Although the Sp1 and GATA-1 binding motifs are necessary elements for hEpoR promoter activity, they are not sufficient to provide erythroid-specific hEpoR expression.

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