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The Gene for Erythropoietin Receptor Is Expressed in Multipotential Hematopoietic and Embryonal Stem Cells: Evidence for Differentiation Stage-Specific Regulation

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The principal regulator of erythropoiesis is the glycoprotein erythropoietin, which interacts with a specific cell surface receptor (EpoR). A study aimed at analyzing EpoR gene regulation has shown that both pluripotent embryonal stem cells and early multipotent hematopoietic cells express EpoR transcripts. Commitment to nonerythroid lineages (e.g., macrophage or lymphocytic) results in the shutdown of EpoR gene expression, whereas commitment to the erythroid lineage is concurrent with or followed by dramatic increases in EpoR transcription. To determine whether gene activity could be correlated with chromatin alterations, DNase-hypersensitive sites (HSS) were mapped. Two major HSS located in the promoter region and within the first intron of the EpoR gene are present in all embryonal stem and hematopoietic cells tested, the intensities of which correlate well with EpoR expression levels. In addition, a third major HSS also located within the first intron of the EpoR gene is uniquely present in erythroid cells that express high levels of EpoR. Transfection assays show that sequences surrounding this major HSS impart erythroid cell-specific enhancer activity to a heterologous promoter and that this activity is at least in part mediated by GATA-1. These data, together with concordant expression levels of GATA-1 and EpoR in both early multipotent hematopoietic and committed erythroid cells, support a regulatory role of the erythroid cell-specific transcription factor GATA-1 in EpoR transcription in these cells. However, the lack of significant levels of GATA-1 expression in embryonal stem cells implies an alternative regulatory mechanism of EpoR transcription in cells not committed to the hematopoietic lineage.

Most of the mature blood cells are short-lived and require constant replenishment from a pluripotent hematopoietic stem cell. Hematopoiesis is thus a cell renewal system in which a constant balance of proliferation and differentiation must be maintained, yet be sensitive to new demands resulting from environmental changes. In vitro studies have identified a group of factors (variously classified as cytokines, colony-stimulating factors, interleukins, lymphokines, etc.) that are necessary for the proliferation, differentiation, self-renewal, and functional activation of the hematopoietic stem cell, its progenitors, and the mature end cell (reviewed in references 2 and 45). While several of these cytokines exert their control primarily on early progenitors, either singly or in concert with other factors, the action of other cytokines is restricted to lineage-committed precursors.

Erythropoietin (Epo) is a glycoprotein cytokine that is the primary regulator of erythroid maturation (reviewed in reference 39). Its biological effect is mediated via a cell surface receptor (EpoR) present in small numbers on normal erythroblasts and erythroleukemia cell lines (reviewed in reference 61). Recently, genomic DNA and cDNA for EpoR have been molecularly cloned from murine erythroleukemia cells (11, 41, 75) and from human fetal liver (33, 73). EpoR shares structural homology with several other receptors of hematopoietic growth factors, lymphokines, and growth hormone-related proteins that belong to a new and growing superfamily of receptors (3).

As Epo is thought to act exclusively on relatively late precursor cells (27), studies aimed at dissecting the regulation of its receptor may help determine its role in commitment and differentiation in the erythroid lineage. We have used cell lines with features of embryonal stem (ES) cells, multipotential hematopoietic progenitors, and erythroid, myeloid, and lymphoid cells arrested at different stages of lineage development to investigate the transcription and chromatin structure of the EpoR gene. These data were also compared with the expression pattern of the erythroid transcription factor GATA-1, previously known as GF-1 (43), NF-E1 (70), or Eryf 1 (16). Taken together, these results show that the EpoR gene is expressed at low levels before either hematopoietic or erythroid commitment has occurred. Commitment to nonerythroid lineages results in the shutdown of EpoR gene expression, whereas commitment to the erythroid lineage is either concurrent with or followed by dramatic increases in EpoR gene expression.

To identify sequences involved in the control of differential activity of the EpoR gene, we have analyzed alterations in chromatin structure as an indicator of regulatory elements. Particular attention was focused on prominent DNase I-hypersensitive sites (HSS) in the first intron that correlated with differentiation stage-specific expression of the gene. These sites are in part associated with GATA-1-binding sequences, and one is unique to cells expressing high levels of EpoR. Transfection studies revealed an erythroid cell-specific and GATA-1-mediated enhancer activity con-

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ferred by this region. Our data thus support a regulatory role of GATA-1 in the erythroid cell-specific control of EpoR gene expression. However, the GATA-1-independent expression of EpoR in cells not committed to the hematopoietic lineage suggests an alternate mode of control.

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MATERIALS AND METHODS

Cell lines. The murine cell lines used in this study are as follows: ES and embryonal carcinoma (EC) cell lines (CCE [15], MBL-1 [52], PCC4 [6], and F9 [6]); hematopoietic multipotent cells from the series FDCP-mix [A4, A7, and 15S(E) (67)]; myeloid progenitors (FDCP-1 and FDCP-2 [12], D35 [25], 32D [26], and WEHI-3B [71]); macrophage cell lines (HA15sus, HA32sus, and HA32 [38]); lymphoid cell lines (CTLL cytotoxic T-cell line [23], E9.D4 helper T-cell line [51], EL4 thymoma [17], LB3 helper T-cell line [36], and ChNS1 B-cell myeloma [9]); and erythroid cell lines (FM2 [22], B8/3, F4N, and F4-12-B2 [40, 50]). Hematopoietic cells and EC cells were maintained in Eagle's minimum essential medium (GIBCO) supplemented with Eagle's nonessential amino acids, twofold essential amino acids, 10% fetal calf serum (or, in the case of FDCP-mix cells, in 20% horse serum), 2 mM glutamine, and, where required, 5 to 10% WEHI-3B conditioned medium as a source of interleukin-3 (IL-3) or 2 to 5% conditioned medium from phorbol ester-induced EL4 thymoma cells as a source of IL-2. LB3 and E9.D4 cells were stimulated weekly with 3,000-rad-irradiated DBA/2 or azobenzenearsonate-coupled CBA spleen cells, respectively. ES cell lines (CCE and MBL-1) were maintained in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 20% fetal calf serum, 10^{-4} M 2-mercaptoethanol, and 2 mM glutamine plus 40% Buffalo rat liver (BRL) conditioned medium as a source of leukemia inhibitory factor.

RNA extraction and analysis. RNA from all cell lines was prepared by the guanidinium isothiocyanate procedure. For Northern (RNA) analysis, the RNA was denatured with glyoxal and dimethyl sulfoxide and size separated by agarose gel electrophoresis. RNA was transferred to Biodyne B (Pall) nylon membrane and hybridized in the presence of 1% sodium dodecyl sulfate, 10% dextran sulfate (LKB-Pharmacia), and 1 M NaCl (60°C, 18 h) with denatured DNA fragments labeled by the method of Feinberg and Vogelstein (18). DNA fragments for probes were obtained from plasmids containing cDNA for EpoR (11), GATA-1 (19b), or β-actin.

Specific RNA transcripts were amplified by the reverse transcriptase-dependent polymerase chain reaction (RT-PCR) (49). RNA (0.01 to 1 μg) was reverse transcribed into cDNA by using avian myeloblastosis virus reverse transcriptase and primed by oligo$(dT)_{12-18}$ (Pharmacia). Specific DNA fragments were subsequently amplified by using *Taq* polymerase (BRL) in a Thermocycler 100 (Braun, Melsungen, Germany), using the conditions originally described by Saiki et al. (59). The amplified product was visualized after separation by agarose gel electrophoresis in the presence of ethidium bromide under UV light. To confirm specificity and to detect low quantities, DNA was transferred to a nylon membrane and hybridized to specific probes as described above. Various cycle numbers and different concentrations of cDNA were tested to ensure a linear range of amplification. RNA isolated from B8/3 cells was used as a standard in all experiments. Quantitative determination was done by densitometric scanning of the autoradiographs.

To determine the copy number of both GATA-1 and EpoR transcripts, known amounts of RNA transcripts synthesized in vitro were used to titrate mRNA levels in B8/3 in RT-PCR assays. Templates used for in vitro RNA synthesis are depicted in Fig. 1. Each construct contains a deletion in the region amplified by the primer pairs such that the resulting amplified DNA is smaller than the expected product from B8/3 cells. In vitro RNA synthesis from the linearized plasmid was carried out in the presence of 0.5 mM nucleoside triphosphates, 10 mM dithiothreitol, 1.5 U of RNasin (Promega), 40 mM Tris-HCl (pH 8.1), 6 mM $MgCl_2$, 2 mM spermidine, and 10 U of T7 RNA polymerase (Boehringer) for 1 h at 37°C in a final volume of 20 μl. Integrity of the RNA was confirmed by gel electrophoresis, and quantity was determined by spectrophotometry. Northern analysis of in vitro-transcribed RNA in parallel with B8/3 RNA confirmed the accuracy of the quantitative determination by PCR analysis.

Protein-DNA binding assays. Nuclear miniextracts were prepared from cells growing in log phase as described by Schreiber et al. (63). All buffers were supplemented with protease inhibitors as previously described (20) and with levamisol as a phosphatase inhibitor (54). Uniform extract quality was checked by assaying at least three replicate preparations for binding of the ubiquitous transcription factor Sp1 (35) to a recognition site in the herpes simplex thymidine kinase (TK) promoter. Some cell lines were also extracted by using the procedure described by Dignam et al. (13) or Gorski et al. (24).

Band shift assays were carried out essentially as described previously (20). A 20-μl reaction mixture contained 4 to 15 fmol of ^{32}P-labeled DNA fragment, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-KOH (pH 7.9), 120 mM KCl, 2 mM $MgCl_2$, 0.5 mM EDTA, 1 mM dithioerythritol, 1.5 μg of poly(dA-dT), 2.5 μg of poly(dI-dC), 0.05% Nonidet P-40, 4% Ficoll, and 8 μl of nuclear extract dilution. Where indicated, a 100- to 200-fold molar excess of specific competitor DNA was added. The end-labeled probe was added after a 15-min preincubation on ice, and the reaction was continued for an additional 15 min. Samples were loaded on a 5% polyacrylamide gel in 0.5× Tris-borate-EDTA and electrophoresed at 10 V/cm.

An oligonucleotide containing sequences from positions −165 to −193 of a −175 HPFH variant of the human γ-globin gene was used as a probe to determine GATA-binding activities:

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        −193                −175        −165
          .                   .           .
5'-TCGAGACACTATCTCAATGCAAACATCTGTCTGAGCT-3'
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This region contains two closely spaced recognition motifs (underlined) and a T→C transition at −175 that strongly reduces the affinity of an overlapping OCT-binding site (29, 43). The probe was titrated with increasing concentrations of nuclear proteins from miniextracts and analyzed by band shift assays. Extracts of different cell lines were standardized on the basis of equal cell numbers. Quantitative determination was done by densitometric scanning of the autoradiographs.

Mapping of HSS. Nuclei from 5×10^7 to 1×10^8 cells were purified essentially as described elsewhere (65), resuspended at $\approx 3 \times 10^7$ cells per ml, and digested with various concentrations of DNase I (Worthington) for 3 min at room temperature. Genomic DNA was then purified, restricted with

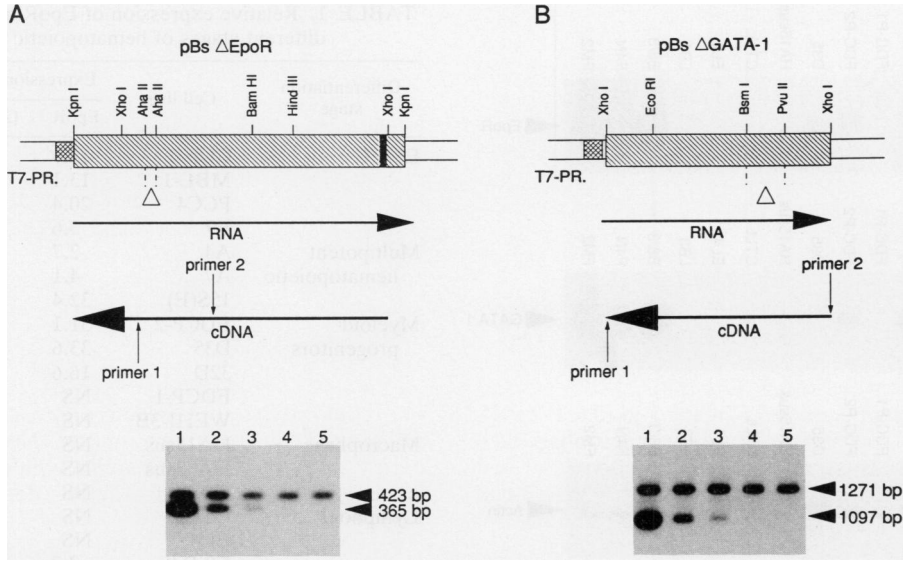

FIG. 1. Quantitative RT-PCR analysis of EpoR and GATA-1 transcription. (A) A *Kpn*I fragment containing an EpoR cDNA in which a 58-bp *Aha*II fragment had been deleted was inserted into pBS (Stratagene) and used as a template for in vitro RNA synthesis. Fivefold dilutions of the resulting transcripts ranging from 5×10^6 to 5×10^4 copies per reaction were reverse transcribed together with 1.5 μg of B8/3 RNA and then amplified by the PCR method (lanes 1 to 5, respectively). Amplimers used were primer 1 (CTGCCAACAGCGGACACATC GAGTTTTGTG [nucleotides 349 to 378]) and primer 2 (GTCCAGGTCGCTAGCGGTCAGTAGTGACGC [nucleotides 742 to 771]) (11) and resulted in a 423-bp fragment for wild-type EpoR cDNA and a 365-bp fragment for the in vitro-transcribed cDNA. (B) A *Xho*I fragment containing a GATA-1 cDNA in which a 174-bp *Bsm*I-*Pvu*II fragment had been deleted was inserted into pBS and used as a template for in vitro RNA synthesis. Fivefold dilutions of the RNA ranging from 5×10^7 to 5×10^5 copies per reaction together with 1.5 μg of B8/3 RNA were subjected to RT-PCR (lanes 1 to 5, respectively). Primers used for GATA-1 amplification were primer 1 ([GTCGAGCTCGAG]C CCATGGATTTTCCTGGTCTAGGG [nucleotides 66 to 89]) and primer 2 ([GTCGAGCTCGAG]CCTTCAAGAACTGAGTGGGGCGAT [nucleotides 1290 to 1313]) (68) and included *Sac*I and *Xho*I restriction sites for cloning. This results in a 1,271-bp fragment for the wild-type GATA-1 cDNA and a 1,097-bp in vitro-transcribed RNA.

either *Eco*RV or *Hin*dIII, and transferred to Biodyne B membranes (Pall) after separation by gel electrophoresis. DNA fragments used as probes for indirect end labeling are indicated in the figure legends. A EpoR genomic clone was obtained by screening a BALB/c genomic library (*Sau*3A partial digest in the EMBL4A lambda vector) with an EpoR cDNA probe as described earlier (31).

Transfection assays. The 951-bp *Eco*RI-*Sma*I genomic DNA fragment (41) corresponding to nucleotides +150 to +1100 (numbering starting at the first coding nucleotide) of the mouse EpoR gene was inserted into the multiple cloning site upstream of the TK promoter of plasmid pBLCAT2 (42). Insertion in either the normal or reverse orientation yielded the construct pERI1(+)/TkCAT or pERI1(−)/TkCAT, respectively. Deletions (5′→3′) of the EpoR sequences to positions +481, +670, and +926 generated plasmids pERI2/TkCAT to pERI4/TkCAT, respectively. In addition, upstream sequences of the EpoR gene from positions −1040 to −5 were fused to a promoterless chloramphenicol acetyltransferase (CAT) reporter gene (pERP-CAT). 3′ extension by sequences containing the first intron of EpoR (*Sst*I fragment extending from positions +640 to +1183) generated plasmid pERPI-CAT.

F4-12-B2, HeLa, and NIH 3T3 cells were transfected at 70% confluence with the various CAT constructs (5 μg/6-cm dish) by using 30 μg of Lipofectin (BRL/GIBCO) according to the method of Felgner et al. (19). In one transfection series, 5 μg of the expression vector pCMV-GATA-1 was cotransfected into NIH 3T3 cells. This vector was generated from murine GATA-1 cDNA obtained by RT-PCR of RNA from FM2 cells. The primers used are given in the legend to Fig. 1B. The amplification product was sequenced to confirm identity with published data, and DNA-binding activity was tested with in vitro transcription/translation products. The cDNA was fused to the enhancer/promoter of the major immediate-early gene of human cytomegalovirus (21), which yielded plasmid pCMV-GATA-1. All cells were harvested 48 h after transfection. Cell harvesting and assay for CAT activity were performed according to the method of Seed and Sheen (64).

RESULTS

Expression of the EpoR gene is not confined to cells committed to the erythroid lineage. To assess expression of the EpoR gene in a wide spectrum of hematopoietic cells representing different stages of lineage commitment, a battery of 23 murine cell lines was chosen. These lines can be grouped into five classes based on differentiation stage: pluripotent ES/EC cells (CCE, MBL-1, PCC4, and F9), multipotent hematopoietic cells (A4, A7, and 15SE), myeloid progenitors (FDC-P2, 32D, D35, FDC-P1, and WEHI-3B), committed lymphoid precursors (LB3, CTLL, EL4, E9D4, and ChNSI), committed macrophage precursors (HA15sus, HA32sus, and HA32), and committed erythroid precursors (B8/3, F4N, and FM2).

Total RNA isolated from these cells was probed for expression of the EpoR gene by Northern analysis (Fig. 2 and data not shown). Not unexpectedly, strong signals were detected in all cell lines representing the erythroid lineage, and no signal was detected in cells committed to either macrophage or lymphoid differentiation. However, tran-

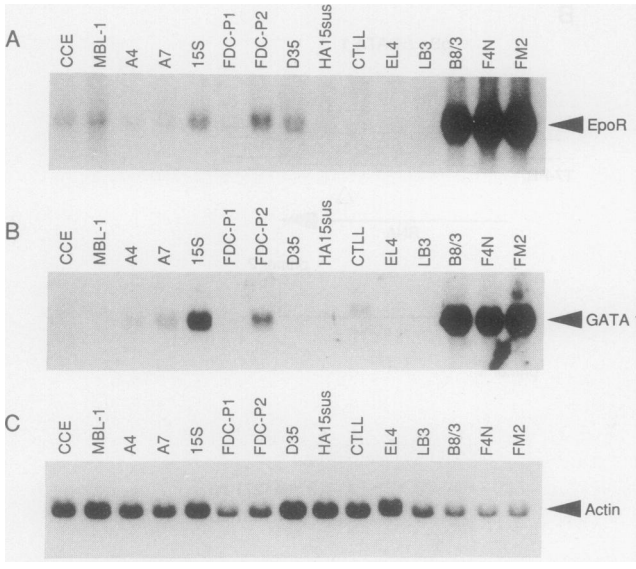

FIG. 2. Expression of EpoR and GATA-1 RNA in various cell lines. To determine levels of EpoR and GATA-1 expression, RNA (15 μg) from several cell lines was subjected to Northern analysis. Filters were sequentially hybridized to cDNA probes for EpoR (A), GATA-1 (B), and β-actin (C). The GATA-1 transcript in CTLL cells was reproducibly larger than that found in other cells. However, the PCR product, which covered the total coding region, had the correct size (not shown). Since no difference in the mobility shift experiments was observed (see Fig. 3), it was tentatively concluded that the GATA-1 transcript in CTLL encodes a normal protein.

TABLE 1. Relative expression of EpoR and GATA-1 mRNAs at different stages of hematopoietic differentiation

Differentiation stage	Cell line	Expression level[a]		Ratio, GATA-1/EpoR[b]
		EpoR	GATA-1	
ES/EC	CCE	12.9	1.6	0.8
	MBL-1	13.1	0.4	0.2
	PCC4	20.4	0.3	0.1
	F9	3.6	0.4	0.7
Multipotent hematopoietic	A4	2.7	6.0	14.4
	A7	4.1	12.4	19.6
	15S(E)	32.4	44.8	8.9
Myeloid progenitors	FDCP-2	31.1	45.2	9.4
	D35	33.6	0.7	0.1
	32D	16.6	3.7	1.4
	FDCP-1	NS	3.3	
	WEHI-3B	NS	NS	
Macrophage	HA15sus	NS	NS	
	HA32sus	NS	NS	
	HA32	NS	NS	
Lymphoid	CTLL	NS	7.0	
	EL4	NS	NS	
	E9.D4	2.5	NS	
	LB3	NS	NS	
	ChNS1	NS	NS	
Erythroid	B8/3	100	100	6.5
	F4N	342	144	2.7
	FM2	563	219	2.5

[a] Relative levels of specific mRNA compared with the B8/3 erythroid cell line were determined by RT-PCR as described in Materials and Methods. The mean of at least two independent experiments is represented as the percentage of B8/3 transcripts. Values less than 0.5 and 0.1% for EpoR and GATA-1 mRNAs, respectively, were considered not significant (NS). This level represents less than 10^4 transcripts per 1 μg of total RNA (approximately 1 transcript per 10 cells).

[b] The ratio of GATA-1 to EpoR transcripts per cell was calculated to 6.5 in B8/3 cells. By quantitative RT-PCR analysis, it was estimated that these cells contain 1.7×10^6 copies of EpoR mRNA and 1.1×10^7 copies of GATA-1 mRNA per 1 μg of total RNA (see Materials and Methods).

scripts were also detected in all cell lines tested that were classified as either ES/EC pluripotent cells or hematopoietic multipotent cells. In addition, some but not all of the myeloid progenitors expressed significant levels of EpoR transcripts. Unlike the cells that we have classified as hematopoietic multipotent cells, these cells have only limited differentiation potential (25, 26) or are blocked in differentiation so that their differentiation potential cannot be tested. These cells most likely represent a wide spectrum of myeloid differentiation in which commitment to one or two lineages may have occurred. Thus, the varied pattern of receptor expression in this group of cells may reflect the different stages of differentiation and commitment.

The more sensitive method of RNA detection, RT-PCR, was also used to confirm results obtained from Northern analysis and to ensure that minor levels of transcription were also detected. Using an amplification protocol that allowed the quantification of specific mRNAs, the level of EpoR transcripts relative to the B8/3 erythroid cell line was determined for all cell lines. The results (Table 1) quantitatively agree with those from Northern analysis. Except for a single case (the E9D4 T-cell line), expression of the EpoR gene is limited to cells of the erythroid lineage or progenitors thereof. Levels in these progenitors are 10- to 100-fold lower than in erythroid cells.

The ratio of GATA-1 to EpoR transcripts varies between cells of distinct differentiation stages. The erythroid transcription factor GATA-1 is thought to be a primary regulator of erythroid cell-specific genes. Since homologies to the consensus GATA-1-binding motif have been found in the EpoR gene region (75), it was of interest to determine whether GATA-1 transcripts were expressed in the same subset of cells that expressed the EpoR gene. Northern blots were rehybridized with a GATA-1-specific probe (Fig. 2), and GATA-1 transcripts in RNA samples were amplified by RT-PCR (Table 1). The ratio between the absolute number of GATA-1 versus EpoR transcripts was determined (Table 1). Interestingly, although GATA-1 transcripts were detected in essentially all of the same cells that expressed EpoR, i.e., hematopoietic cells committed to the erythroid lineage or cells that represented progenitors of the erythroid lineage (e.g., stem cells), a significant difference was found in the ratio between GATA-1 and EpoR expression in cells of disparate differentiation potentials. This result was in contrast to relatively constant ratios found between cells of similar differentiation stages. For instance, the mean ratio of GATA-1 to EpoR transcripts in ES/EC cells was 0.45 ± 0.35, in contrast to a mean ratio of 14.3 ± 5.4 for multipotent hematopoietic cells, a statistically significant difference (t test, $P = 0.1$). A somewhat lower value was found in cells committed to erythroid differentiation ($\bar{x} = 3.9 \pm 2.3$). A wide variation in the ratio of GATA-1 to EpoR expression in cell lines classified as myeloid progenitors was found (ranging from 0.1 to 9.4), which again may reflect the wide scope of differentiation potentials of this group. These results suggest that the GATA-1 and EpoR genes can be independently expressed and that distinct ubiquitous and differentiation-specific factors must regulate their expression in different cells.

Correlation of GATA-1 protein levels with mRNA levels in

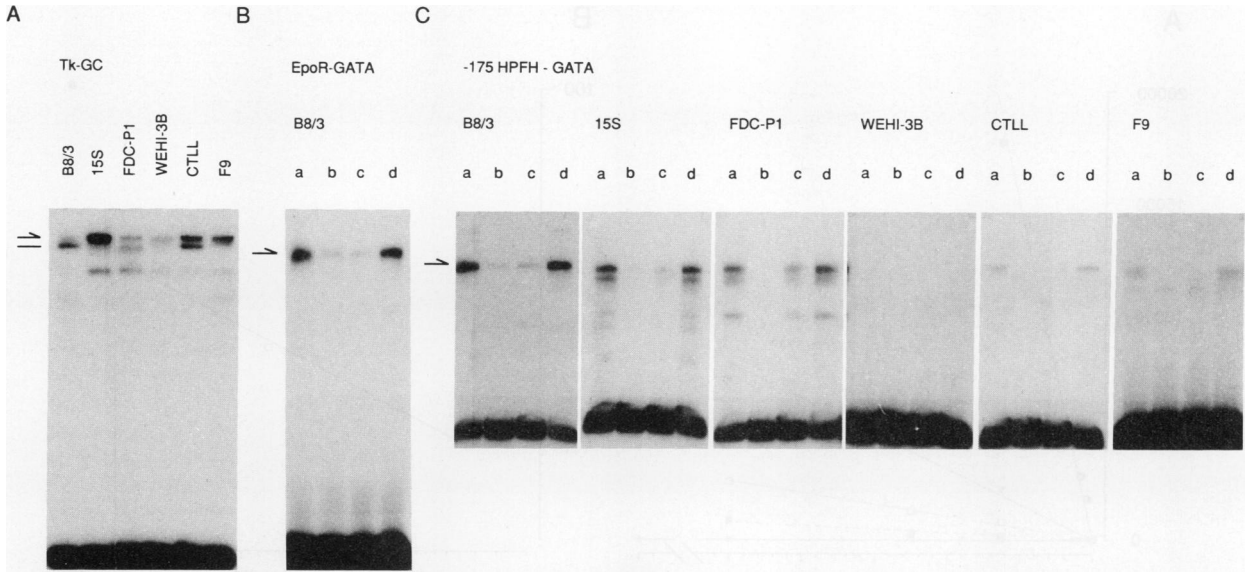

FIG. 3. Analysis of tissue distribution and binding specificities of GATA-binding activities by mobility shift experiments. (A) Nuclear miniextracts were prepared as described in Materials and Methods. General assay quality was checked by binding of the ubiquitous transcription factor Sp1 to an oligonucleotide (TK-GC) containing a GC box of the TK promoter (positions −109 to −81). (B and C) Competition band shift assays were performed with oligonucleotides with GATA consensus motifs from either the EpoR promoter (EpoR-GATA; −206 to −189 [75]) (B) or the γ-globin promoter (−175 HPFH-GATA; −193 to −165) (C). Nuclear proteins of the various cell lines were bound to the labeled probes under standard assay conditions (lanes a) or in the presence of an excess of the following competitor sequences: −175 HPFH-GATA (lanes b), EpoR-GATA (lanes c), or TK-GC (lanes d).

various cells. To test whether the GATA-1 mRNA detected by PCR was indeed translated into protein, specific DNA binding was analyzed as a measure for functional GATA-1. Nuclear extracts were prepared from equal numbers of cells from selected cell lines. Extract quality was checked by binding of the ubiquitous transcription factor Sp1 to a cognate site in the TK gene promoter. All extracts tested showed in various proportions two retarded bands in the gel shift assay (Fig. 3A) representing differently glycosylated isoforms of Sp1 (32). Although ubiquitously expressed, Sp1 showed a remarkably uneven distribution in the different cell lines, in agreement with results of others (58).

Using extracts from the B8/3 erythroid cell line as a source of GATA-1, we observed strong binding to the recognition sequence of the γ-globin gene promoter that was originally used as a probe to identify a GATA-1 cDNA (68) (Fig. 3C). A consensus sequence found in the EpoR promoter (75) was bound with somewhat lower affinity (Fig. 3B). Both DNA motifs showed effective cross-competition, indicating homologous binding characteristics. A heterologous sequence motif did not interfere with binding. We then tested extracts from nonerythroid cell lines (F9, 15S, FDCP-1, CTLL, and WEHI-3B) which were expected by the PCR data to contain either no or differing levels of GATA-1. In all cases where anticipated, a band with the same mobility as GATA-1 from B8/3 could be detected (Fig. 3C). In some extracts, additional bands with lower mobility were detected. These bands may represent degradation products, although more demanding procedures for extract preparations (see Materials and Methods) did not change the pattern. However, as DNA interactions with extracts of FDCP-1 cells were only weakly competed for by the GATA motif of the EpoR promoter, we interpreted this as binding of different proteins with related recognition characteristics. Additional members of a multigene family that recognize the GATA consensus motif have been identified (72, 74).

Using the criteria of mobility and competition characteristics found with GATA-1 of B8/3 for specific GATA-1 DNA-binding activity, a qualitative agreement between the occurrence of GATA-1 mRNA and protein was found. A comparative determination of GATA-1 levels in those nuclear extracts that allowed an unambiguous assignment by the above criteria showed that the nonerythroid cell lines had a 10- to 100-fold-lower abundance of GATA-1 than did the highly expressing erythroid cell line B8/3 (Fig. 4A). Interestingly, the DNA-binding activities of GATA-1 correlated linearly with the mRNA levels determined by PCR for the nonerythroid cell lines, whereas B8/3 contained substantially more protein in relation to mRNA (Fig. 4B). This finding suggests that a control mechanism acting at the level of either translation or protein stability may exist in erythroid cells for the accumulation of GATA-1.

Alterations in chromatin structure of the EpoR gene locus reflect expression levels at distinct differentiation stages. Chromosomal regions engaged in the control of gene activity are generally characterized by an open chromatin configuration which allows access of *trans*-acting factors (reviewed in reference 28). These regions are operationally defined by their hypersensitivity to DNA-modifying chemicals or enzymes such as endonucleases. To correlate gene activity as determined by RNA analysis with structural alterations in the chromatin of the EpoR gene region, we examined the sensitivity of this locus to digestion by DNase I in a selected set of cells. To this end, genomic clones containing the entire EpoR transcribed region and flanking sequences were isolated from a BALB/c genomic library. A restriction map of the resulting clone containing the EpoR gene locus is depicted in Fig. 5, along with probes used for mapping HSS.

HSS within the EpoR locus of cells of the erythroid lineage (B8/3 and FM2) were first mapped by using three probes that hybridized to either of two *Eco*RV fragments or to a *Hin*dIII fragment. Using this combination, we were able

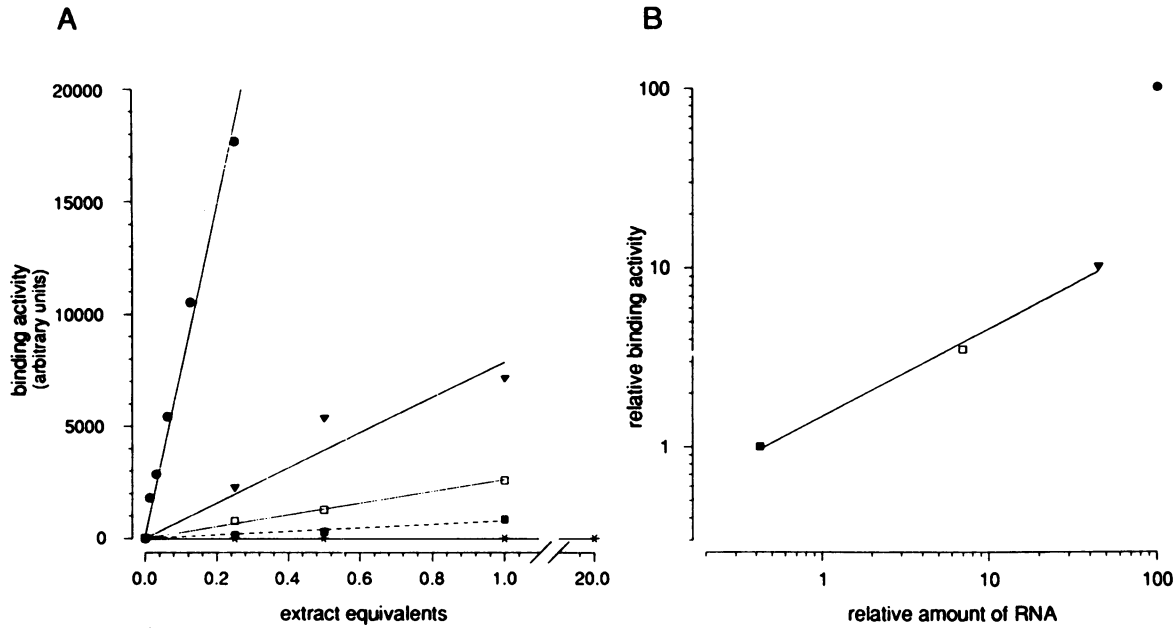

FIG. 4. DNA-binding activities and mRNA levels of GATA-1 in various cell lines. (A) Titration of GATA-1 interaction with the high-affinity binding motif of the γ-globin promoter (−175 HPFH-GATA). The probe was incubated with increasing amounts of nuclear extracts from B8/3 (circles), 15S (triangles), CTLL (open squares), F9 (closed squares), and WEHI-3B (asterisks) cells. Extract equivalent to 4×10^5 cells was assigned the value of 1. Binding activities were determined as described in Materials and Methods. (B) Correlation of DNA-binding activities and mRNA levels in B8/3, 15S, CTLL, and F9 cells (symbols as in panel A). Binding activities were derived from the slopes in panel A and normalized with respect to B8/3 activity, which was arbitrarily set at 100. RNA levels determined by RT-PCR were taken from Table 1 and also expressed as amounts relative to B8/3.

to detect a total of nine sites within 14 kb of the EpoR locus. The most prominent site was detected as a 1.6-kb degradation band from the 9.0-kb *Eco*RV fragment (Fig. 6A) or a 1.8-kb degradation band of the 4.3-kb *Hin*dIII fragment (Fig. 7) and is depicted in Fig. 8 as HSS 5. Two other less prominent but still major sites were also mapped from the 9.0-kb *Eco*RV fragment and the 4.3-kb *Hin*dIII fragment and are depicted in Fig. 8 as HSS 3 and HSS 6; the latter site mapped within 100 bp of the strong HSS 5 site. An additional six HSS could be detected that, although consistently present, were more diffuse and most likely represent minor sites. All HSS were clustered in a region ranging from around positions −1200 to +1800. Two of the major sites (HSS 5 and HSS 6) were located within the first intron, and

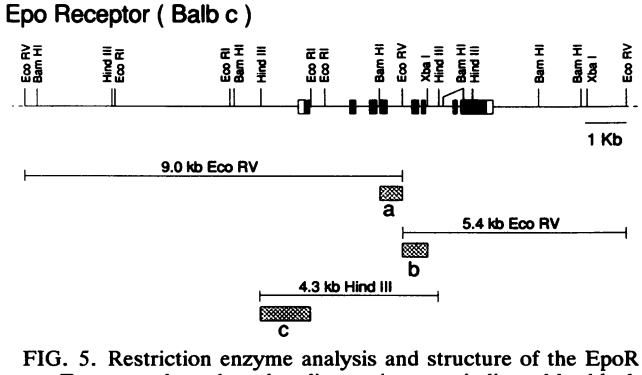

FIG. 5. Restriction enzyme analysis and structure of the EpoR gene. Exons are boxed, and coding regions are indicated by black shading. Restriction fragments and probes used for mapping HSS are indicated.

the third (HSS 3) was located in the promoter region. No HSS were readily detectable downstream of the transcribed region (Fig. 6B and 8).

Results obtained from cells that expressed moderate levels of EpoR (the EC cell line PCC4 or the multipotent hematopoietic cell line 15S) showed a strikingly different pattern. Only two HSS, HHS 3 and HHS 6, could be detected, both of which corresponded to one of the three major sites found in the erythroid cells B8/3 and FM2 (Fig. 7 and data not shown). Faint levels of HSS 8 were also detected in these cells. Significantly, although the sensitivity to DNase I of the two major sites corresponded to that in the erythroid cells, the prominent HSS 5 found in all erythroid cells tested was not present. Although its close proximity to HSS 6 may render an unequivocal assignment difficult, repeated digestions and probing from both directions confirmed our conclusion that it was present only in cells of the erythroid lineage (Fig. 7).

The EpoR locus in cells that expressed only minor levels of transcripts (e.g., the multipotent hematopoietic cell A4) also contained only two clear sites, corresponding to the HSS 3 and HSS 6 also found in PCC4 and 15S cells (Fig. 7). These were, however, much reduced in intensity. Similarly, in cells expressing no significant levels of EpoR mRNA (e.g., the lymphoid cell line ChNS1 and the myeloid progenitor WEHI-3B), both the major HSS 3 and HSS 6 as well as the minor HSS 8 could be detected at high concentrations of DNase I (Fig. 6A and B and data not shown). These sites were quite weak and not always detectable.

In summary, our mapping of HSS as an indicator of local points of gene regulation of the EpoR locus has detected three major and six minor HSS in erythroid cells. Of the three major sites, one (HSS 5) is strikingly pronounced and

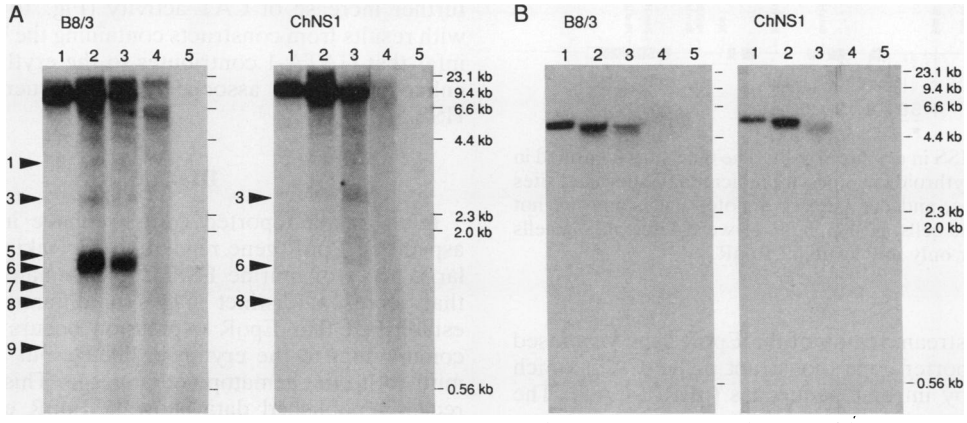

FIG. 6. Mapping of HSS in B8/3 and ChNS1 cells. DNase I-treated genomic DNA (15 μg) was digested with *Eco*RV and probed with either probe a (A) or probe b (B) as shown in Fig. 5. DNase I concentrations were as follows: lane 1, no DNase I; lane 2, 1.8 μg/ml; lane 3, 2.7 μg/ml; lane 4, 4 μg/ml; and lane 5, 6 μg/ml. All major and minor degradation bands of either the 9.0- or 5.4-kb fragment are indicated and numbered according to the resulting map presented in Fig. 8.

unique to erythroid cells in which high levels of EpoR mRNA transcripts have been detected. In all other hematopoietic or ES/EC cells examined, only two major HSS (HSS 3 and HSS 6) are present. The intensities of these sites are directly proportional to the transcriptional activity of the EpoR gene.

HSS 5 and HSS 6 map to a regulatory domain with erythroid specificity. To prove that the sequences underlying the prominent HSS 5 and HSS 6 harbored transcriptional control elements, we conducted a series of transfection assays. Figure 9 presents sequence data for the first intron of EpoR (41; our results) and includes a delineation of the regions containing HSS 4 to HSS 7 as well as the consensus binding sites for *trans*-activators as determined by a homology search. A genomic fragment containing the entire first intron was placed in front of a TK-CAT fusion construct and assayed for transcriptional enhancement. In the adherent erythroid cell line F4-12-B2, the inclusion of EpoR sequences in the parental construct resulted in a sevenfold increase in CAT activity, independently of orientation (Fig. 10). EpoR sequences imparted no or only a weak enhancement in nonerythroid cell lines (HeLa and 3T3), which appeared to be orientation dependent. A deletion that removed 5′ sequences of the intron including HSS 4 but maintained HSS 5 to HSS 7 (Fig. 9) did not result in decreased activities. Deleting additional sequences containing the closely spaced HSS 5 and HSS 6 significantly reduced enhancement. Since a sequence inspection revealed potential GATA-1-binding sites in the 3′ half of the intron region, we assessed their regulatory significance by transfection studies into nonerythroid cells which ectopically expressed GATA-1. When construct pERI3-TkCAT was cotransfected into NIH 3T3 cells with an expression vector coding for GATA-1, a moderate stimulation of CAT activity was observed, whereas construct pERI4-TkCAT was unresponsive (Fig. 10). This result suggested that GATA-1 interaction with recognition sites in the HSS 5 region may contribute to erythroid specificity.

Transcriptional enhancement by *cis* elements within the first intron was also tested in the context of the homologous

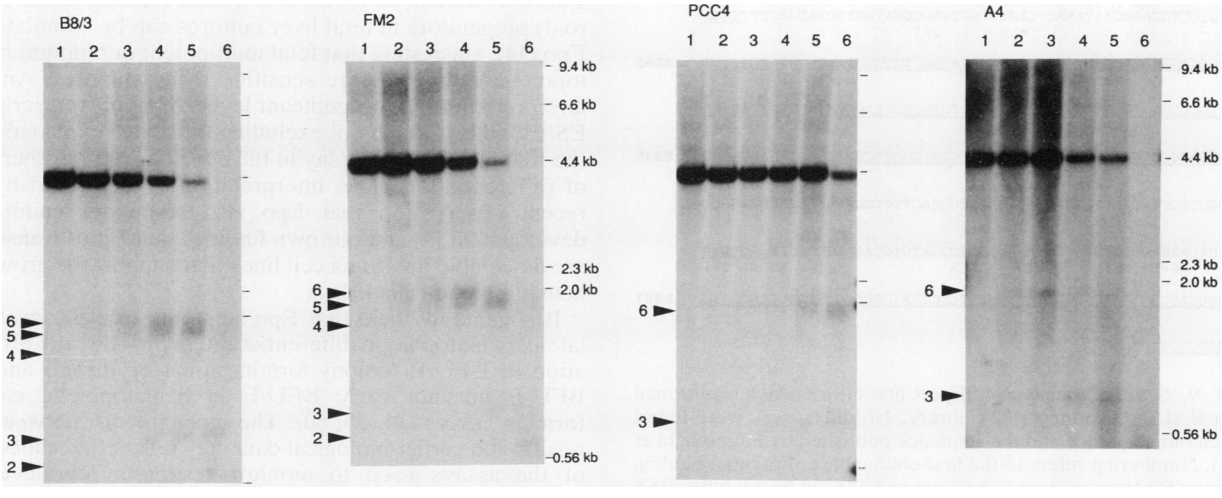

FIG. 7. Comparison of HSS in different cell lines. DNase I-treated genomic DNA (10 μg) was digested with *Hin*dIII and probed with probe c as shown in Fig. 5. DNase I concentrations were as follows: lane 1, no DNase I; lane 2, 0.8 μg/ml; lane 3, 1.2 μg/ml; lane 4, 1.8 μg/ml; lane 5, 2.7 μg/ml; and lane 6, 4 μg/ml. Major and minor degradation bands of the 4.3-kb fragment are indicated and numbered according to the map presented in Fig. 8.

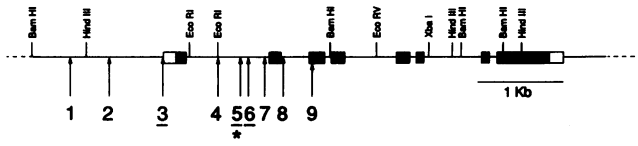

FIG. 8. Map of HSS in erythroid cells. The nine sites identified in the B8/3 and FM2 erythroid cell lines are indicated. Underlined sites represent major sites, and the asterisk denotes the major site not found in nonerythroid cells. HSS 3, 6, and 8 were detectable in cells that expressed no or only low levels of EpoR.

promoter. The upstream region of the EpoR gene was fused 5′ to the CAT reporter gene (construct pERP-CAT) which was extended 3′ by intronic sequences (pERPI-CAT). The two constructs showed the same basal activity when transfected into NIH 3T3 cells. Coexpression of GATA-1 resulted in a fivefold stimulation of the EpoR promoter. Similar results have recently been reported with use of COS 7 cells as recipients (8). From deletion analyses, it was concluded that *trans* activation was mediated mainly by a GATA motif situated around position −200. (The same motif was used in our band shift competition assays; Fig. 3.) The addition of intronic sequences as in construct pERPI-CAT resulted in a

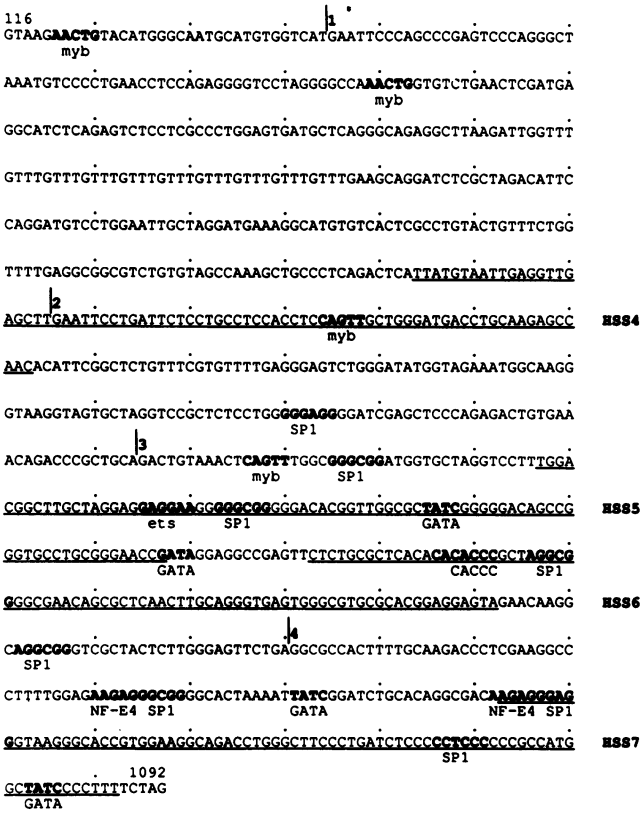

FIG. 9. Sequence analysis of EpoR first intron which was cloned from a BALB/c genomic DNA library. No differences were found between our sequence and the sequence published by Kuramochi et al. (41). Numbering refers to the first coding base. Potential binding sequences for transcription factors are specified in heavy type. HSS could be defined with an accuracy of ±40 bp and are underlined. The 5′ endpoints of the deletion variants are indicated by vertical lines and numbered 1 to 4 in reference to constructs pERI1-TkCAT to pERI4-TkCAT.

further increase of CAT activity (Fig. 10). In conjunction with results from constructs containing the TK promoter, we infer that GATA-1 contributes to the erythroid cell-specific enhancer activity associated with sequences surrounding HSS 5.

DISCUSSION

In the work reported here, we have investigated three aspects of EpoR gene regulation. By taking advantage of a large array of murine ES/EC and hematopoietic cell lines that represent distinct stages of differentiation, we have established that EpoR expression occurs not only before commitment to the erythroid lineage but also before commitment to the hematopoietic lineage. This finding confirms recently published data showing EpoR expression in ES cells (62). Second, we have ascertained that EpoR mRNA expression is not necessarily accompanied by GATA-1 mRNA expression, suggesting common as well as independent regulatory mechanisms. Third, in assessing potential cellular mechanisms for transcriptional control, we have been able to define a region within the first intron of the EpoR gene that contributes to the high erythroid expression of this gene.

The expression of the EpoR gene in both early hematopoietic progenitors and ES cells has interesting implications for the role of Epo in early development and hematopoietic differentiation. Although the levels of EpoR expression are lower in both ES/EC and early hematopoietic progenitors than in erythroid precursors, the distinctive chromatin structure of the EpoR gene in these cells supports the conclusion that expression is not limited to a small minority of spontaneously differentiated cells. The relative high levels of EpoR expression in ES/EC cultures are especially intriguing in light of our findings that the receptor for IL-3, a cytokine that acts on early multipotent hematopoietic cells, is not expressed in these cells (30a). One possible explanation is the predominate role that Epo may play in embryonic and fetal hematopoiesis. In the mouse embryo, erythropoiesis is first detected in the yolk sac at day 7.5 of gestation, whereas other differentiation lineages (e.g., macrophages and mast cells) are detected 2 to 3 days later (46, 66). Interestingly, studies have also shown that unlike in adult bone marrow or spleen cultures, early BFU-E (burst-forming units–erythroid) progenitors in fetal liver cultures can be stimulated by Epo (14), suggesting that fetal multipotent or bipotent hematopoietic cells are more sensitive to its stimulus. Another interpretation of the significant levels of EpoR transcripts in ES/EC cells, though not excluding the above explanation, is the role that Epo may play in the development of other lines of differentiation. This interpretation is supported by the recent observation that Epo also stimulates endothelial development (1) and our own findings that EpoR transcripts are detectable in stroma cell lines that support the growth of hematopoietic cells (31b).

It is generally held that Epo acts only on cells relatively late in hematopoietic differentiation, supporting the proliferation of CFU-E (colony-forming units–erythroid) and late BFU-E but not early BFU-E in hematopoietic colony-forming assays (14, 27, 60). The incongruence between our results and earlier biological data may reflect the inadequacy of the assays used to monitor response, low levels of functional EpoR, or nonfunctional receptors. Significantly, all of the myeloid progenitor cell lines used in this study that are blocked in differentiation but positive for EpoR transcripts can be stimulated by Epo to proliferate in at least

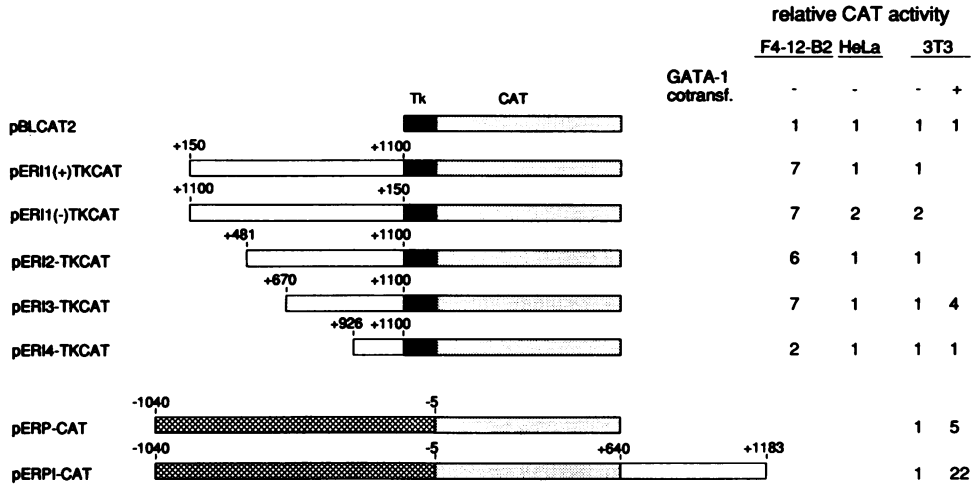

FIG. 10. The first intron of the EpoR gene contains regulatory elements with erythroid specificity. A 951-bp fragment containing the first intron of the EpoR gene from positions +150 to +1100 (numbering refers to the first coding base [41]) was ligated in front of a TK-CAT fusion construct (pBLCAT2) in either the natural [pERI1(+)TkCAT] or inverse [pERI1(−)TkCAT] orientation. In addition, 5′→3′ deletions of the intronic sequences were fused to the basic plasmid. In a second set of constructs, the TK promoter was replaced by upstream sequences of the EpoR gene extending from −1040 to −5 (pERP-CAT). 3′ fusion of intron sequences generated plasmid pERPI-CAT. These constructs were transfected into the erythroid cell line F4-12-B2 or into nonerythroid cell lines (HeLa and NIH 3T3), and CAT activities were determined. A subset of constructs was cotransfected into NIH 3T3 cells with the expression vector pCMV-GATA-1. Control transfections received the same amount of vector pCMV-SEAP, coding for the secretion protein SEAP (5) instead of the *trans*-activator GATA-1. The activities of the parental constructs were set at 1. The data represent means of two experiments which do not deviate by more than 22%.

short-term assays (41a). The immediate induction of differentiation that occurs when IL-3 is removed in the multipotent hematopoietic cells (A4, A7, and 15S) used in this study does not allow identification of the target cells of Epo stimulation; nonetheless, it is tempting to speculate that early hematopoietic cells do express an active EpoR which can at least transmit a proliferation signal, either alone or in synergism with other cytokine receptors. We find it unlikely that the stimulation of EpoR in these early cells results in erythroid commitment in light of several recent studies that have investigated the differentiation-inducing activity of another lineage-specific cytokine, macrophage colony-stimulating factor 1, and its receptor (c-*fms*) in either multipotent hematopoietic cells (37, 53) or restricted bipotent cells (7, 56). These studies have demonstrated that innate properties of the cell itself influence dramatically the action of the activated receptor. A number of mechanisms may be envisaged that would inhibit differentiation induction by the ligand in these early progenitors, including down-modulation by other hematopoietin receptors (34, 69), the absence of necessary components of the signal transduction system (37), or lack of transcriptional competency of hormone-responsive genes. Only when a progenitor cell has established all permissive conditions can one of the growth factors be instructive. It is conceivable that at a particular stage of differentiation (perhaps representing the late BFU-E), activation of EpoR could trigger events leading to erythroid maturation. This differentiation program could be accompanied or followed by increased transcription of the EpoR gene. It is of special interest that differentiation commitment to nonerythroid lineages results in the shutdown of EpoR transcription. This down-regulation does not appear to be at the level of chromatin structure, as the EpoR gene in cells that do not express EpoR mRNA is still sensitive to DNase I, albeit distinct HSS are less prominent than in cells expressing moderate levels of EpoR.

What role does GATA-1 play in the regulation of the EpoR gene expression and in erythroid commitment? Significantly, both GATA-1 and EpoR are expressed in early multipotent hematopoietic progenitors. Furthermore, similarly to the EpoR gene, commitment to the erythroid lineage is accompanied by large increases of GATA-1 expression, whereas differentiation to a nonerythroid lineage, e.g., lymphoid or macrophage, results in a complete shutdown of expression. Expression of GATA-1 in megakaryocytic and mast cell lineages (44, 57) and in the myeloid progenitor cell line 32D (10) has been recently reported; however, these studies did not examine cells with earlier differentiation potentials. Although GATA-1 was originally postulated to be erythroid cell specific and thus a potential master switch of erythroid commitment, our results clearly indicate that the presence of this transcription factor is not sufficient to induce commitment to a particular lineage. The correlation between GATA-1 and EpoR expression during hematopoietic and erythroid differentiation found in our study suggests either common controls or a regulatory role of GATA-1 for EpoR expression. That GATA-1 is a regulator of EpoR expression is supported by transfection experiments using deletion mutations of the EpoR promoter (8) and by our analysis of the sequences surrounding a prominent HSS specific for the erythroid lineage. This HSS is located in the first intron, and sequences surrounding this site conferred significant erythroid cell-specific activity to a heterologous promoter. Sequence analysis revealed the occurrence of two GATA-1 consensus motifs within this HSS, and this region mediated a GATA-1-dependent response in nonerythroid cells which ectopically expressed the activator. Of the four GATA-1 consensus motifs within the first intron (Fig. 9), we have examined two by mobility shift assays (19a). Competition binding revealed a moderate to low affinity, and from sequence homology we infer a similar binding strength for the two others. We therefore imagine that GATA-1 has to interact with other transcription factors to elicit a response. Interestingly, potential binding sites for both *myb* and the *ets*

family of DNA-binding proteins are located near the erythroid cell-specific HSS 5. Both transcription factors have been implicated in erythroleukemic transformation (4, 47, 55), and more recently *myb* expression has been shown to be necessary for normal hepatic erythropoiesis (48). Preliminary footprinting data of this region have shown multiple protein-DNA interactions in the 3′ half of the first intron; however, the exact nature of the cognate factors has yet to be determined. Nevertheless, this finding indicates that the region encompassing HSS 5 to HSS 7 constitutes a complex regulatory domain.

A recent finding suggested that GATA-1 transcription may also be controlled in part by GATA-1 (30). Interestingly, this gene also contains regulatory elements in the upstream transcribed region including the first intron. In light of these data, it is tempting to speculate that the coupled expression of GATA-1 and EpoR during hematopoietic and erythroid differentiation reflects shared regulatory elements, part of which is GATA-1. The interaction of these two proteins in the control of their expression during hematopoietic differentiation is of particular interest and awaits a more thorough understanding of their regulation.

Although a correlation between EpoR and GATA-1 expression could be found in hematopoietic cells, the lack of significant levels of GATA-1 transcripts in ES/EC cells precludes a regulatory role of GATA-1 in EpoR transcription in all cells. In accord with the hypothesis that the relative high levels of EpoR expression in these cells is due in part to its capacity to stimulate development along other lineages, these results are consistent with an alternative mode of regulation in nonhematopoietic cells. Notably, no GATA-1 transcripts are found in stromal cell lines that express significant levels of EpoR (31a).

The study presented here provides a basis for designing new experiments in which the role that EpoR and GATA-1 play in both hematopoietic and erythroid differentiation can be addressed. Analysis of the critical events, such as the up- and down-regulation of their expression during differentiation and commitment, is indispensable in our understanding of the controls that regulate the necessary balance between differentiation and self-renewal in hematopoiesis.

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