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GATA-1 transactivates erythropoietin receptor gene, and erythropoietin receptor-mediated signals enhance GATA-1 gene expression

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ABSTRACT

Erythropoietin is a cytokine which specifically regulates the proliferation and differentiation of erythroid progenitor cells. The expression of erythropoietin receptor on the cell membrane of the progenitor cells is a critical event during the erythroid differentiation process. In order to clarify the tissue-specific and differentiation stage-specific expression of the erythropoietin receptor gene, its transcriptional regulation was examined by transient expression assay, gel mobility shift assay and DNase I footprinting. The results clearly showed that GATA-1 transactivates the gene expression through a single GATA motif located around −200 bp upstream from the ATG codon in a dose dependent manner. Furthermore, Northern blot analysis revealed that erythropoietin receptor-mediated signals strongly enhanced GATA-1 gene expression in accordance with the appearance of hemoglobin-positive cells. Taken together with other observations, these results suggested the following scheme of erythroid differentiation: 1)GATA-1 is expressed in the early stage of blood cell development; 2) GATA-1 transactivates the erythropoietin receptor gene; 3) erythropoietin binds its receptor and the receptor-mediated signals enhance GATA-1 gene expression in erythroid progenitor cells; and 4) GATA-1 finally transactivates hemoglobin synthesis-related genes and globin genes in relatively matured erythroid cells.

INTRODUCTION

Erythropoietin (Epo) is a glycoprotein factor which specifically acts on erythroid progenitor cells and stimulates their proliferation and differentiation (1). During erythroid differentiation, the receptors for Epo (EpoR) are specifically expressed in the erythroid burst forming unit (BFUe) and the erythroid colony forming unit (CFUe); cells expressing the receptor thus respond to the circulating Epo, and proliferate and differentiate into mature erythrocytes.

Recently, cDNAs encoding mouse EpoR were cloned and characterized (2,3). Analysis of cDNA structures revealed the existence of two classes of cDNA produced by alternative splicing: one encodes a membrane-bound receptor, and the other a soluble receptor lacking the transmembrane and cytoplasmic domains (3). In an attempt to clarify the molecular mechanism of erythroid-specific and differentiation stage-specific expression of the EpoR gene, we have isolated the mouse genomic EpoR gene (3,4). Through the analysis of 6,6 Kb genomic DNA segments covering the complete EpoR gene and promoter regions, it was found that five putative GATA motifs and an Sp1 binding site but neither TATA box nor CAAT box exist in 1,7 Kb 5′-flanking regions (3).

An erythroid-specific nuclear factor regulating α and β-globin gene expression has been identified and designated GATA-1 (previously known as NF-E1, GF-1 or Eryf-1)(5−11). GATA-1 is a 413 amino acid polypeptide that contains two finger-like DNA binding domains. GATA-1 binds to consensus sequences (WGATAR or YTATCW) which are present in promoter or enhancer regions of erythroid-specific genes such as globin genes, porphobilinogen deaminase gene and carbonic anhydrase I gene, and transactivates these gene expressions in relatively matured erythroid progenitor cells (12−15). There is no doubt that GATA-1 is required for normal erythroid differentiation, since disruption of this gene by homologous recombination resulted in a lack of mature red blood cells in chimaeric mice (16). Thus GATA-1 may be the primary determinant protein of erythroid lineage, and may regulate the expression of various erythroid-specific genes during the development of blood cells. The exact stage in the erythroid lineage at which GATA-1 exerts its effect, however, has not yet been identified. GATA-1 gene expression may not be activated until EpoR-mediated signals are transmitted, or may be expressed before EpoR appears on the cell membrane and may transactivate the EpoR gene to trigger the erythroid lineage. In the latter case, EpoR gene might be one of the earliest target genes of GATA-1, and might be expressed specifically

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in BFUe and CFUe prior to gene expression of hemoglobin synthesis-related enzymes.

To investigate the molecular mechanism of erythroid differentiation, we examined the possible role of GATA-1 in the regulation of EpoR gene expression, and the effects of EpoR-mediated signals on GATA-1 gene expression during this differentiation.

MATERIALS AND METHODS

Cell culture

Mouse erythroleukemia SKT6 cells have been described previously (17), and were maintained in Ham's F-12 medium containing 10% defined fetal calf serum (Cell Culture Lab.). The cells were induced to differentiate by the addition of 0.5 unit/ml of recombinant human Epo (over 70,000 units/mg; Kirin Brewery, Tokyo). COS 7 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum.

Construction of plasmids

For the construction of pERcat plasmids, BamH I-Hae II fragment (−1708 to −38 bp relative to ATG codon) of pMGER which encodes the entire mouse genomic erythropoietin receptor gene (3) was prepared. Double-stranded oligonucleotides (5′-TGGAGCCCTGAGCTTCCTGAAGCTAGGCTGCATCA-3′/3′-CGCGACCTCGGGACTCGAAGGACTTCGACCCGACGCTAGTTCGA-5′) corresponding to Hae II-Nla III fragment (−38 to 0 bp relative to the ATG codon) were synthesized. pSV00CAT was digested with Hind III, and ligated with the above two DNA fragments, of which BamH I and Nla III sites were changed to Hind III sites. To confirm that the synthesized sequences and the promoter orientation were correct, about 350 bp sequences from both Hind III sites were sequenced, and the constructed reporter plasmid was designated p1.7ERcat. To construct the deletion mutants, 1.7 kb Hind III fragment of p1.7ERcat was digested with Nde I, Sph I and Hae III, respectively; the resulting 1.6 kb, 0.6 kb, 0.18 kb DNA fragments were blunt-ended, ligated with pSV00CAT using Hind III linker, and designated p1.6ERcat, p0.6ERcat and p0.2ERcat, respectively.

Mouse GATA-1 cDNA from erythroleukemia SKT6 cells was obtained by the polymerase chain reaction using synthesized 5′ primer (5′-CAAGCCCAGGTTCAACCCCAGTGTTCCCATG-3′) and 3′ primer (5′-AGGCTATTCTGTGTACCTTCAAGAACTGAG-3′). The isolated GATA-1 cDNA was sequenced by M13 dideoxy chain termination method, and confirmed to be identical with that previously reported (8). The blunt-ended cDNA was inserted into blunt-ended Pst I and BamH I sites of pcDSRα296 (18), and designated pSRαGATA-1.

Transfection and CAT assay

Approximately 10^6 COS 7 cells were plated on 10 cm dishes 24 hr before transfection. Two hours before transfection, the culture medium was replaced and cells were transfected by calcium phosphate co-precipitation (19) with 1.1 pmol of the reporter plasmid (e.g., 5 μg for p1.7ERcat), 2 μg of reference plasmid pCH110 (coding for β-galactosidase) with or without 7 μg of pSRαGATA-1. After 8 hr exposure to the precipitates, a glycerol shock was applied for 2 min, and cells wee rinsed with PBS and refed with culture medium. All transfections were performed in duplicate and in three independent experiments for each construct. Cells were harvested after 60 hr incubation, and lysed by sonication. Cleared lysates were assayed for β-galactosidase activity using chlorophenol red-β-D-galactopyranoside (Behringer) as substrate. The measured activities were used to correct the amount of extract used in the CAT assay for the variation in transfection efficiency. CAT assays were performed as described (19). The acetylated and non-acetylated forms of chloramphenicol were separated by TLC, visualized and quantitated by an imaging analyzer (Fujix, BAS 2000 system).

Gel mobility shift assay

Nuclear extracts of SKT6 and COS 7 cells were prepared by the method of Dignam *et al.*(20). Protein concentrations were determined by the method of Bradford (21). A 94 bp DNA fragment (−215 to −122 bp region) containing a GATA motif and anSp1 binding site was prepared by EcoT14 I and Msp I digestion of 0.6 kb Hind III fragment derived from p0.6ERcat, and end-labeled using Klenow enzyme and [^{32}P]deoxynucleotides. The DNA fragment (1 ng) was incubated at room temperature for 20 min with 3 μg of nuclear extracts in the presence or absence of 100 ng unlabeled DNA, in 20 μl of binding buffer consisting of 10 mM Tris-HCl pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, 5% glycerol and varying amounts of the carrier polymer poly(dIdC). The DNA-protein complexes were then separated in 4% polyacrylamide gel (acrylamide to bisacrylamide ratio of 30:1) containing 40 mM Tris-acetate (pH 7.8) and 2 mM EDTA. Gels were run at 100 V for 7 hr in a cold room, and then dried prior to autoradiography.

DNase I footprinting

DNase I footprinting studies were done with the 3′ end-labeled 94 bp DNA fragment. One end-labeled DNA fragment (90 ng) was incubated with 270 μg of SKT6 or COS 7 nuclear extracts in 1.8 ml of binding buffer (600 μl reaction mix per tube) for 20 min at 20 C. $CaCl_2$ was added to 1 mM, and the samples were then digested with 0.003−0.007 unit of DNase I (Worthington Diagnostics) for 1 min at room temperature. A control reaction, containing no nuclear extracts but the same concentration of bovine serum albumin, was also digested with DNase I under the same conditions. The reactions were stopped with the addition of EDTA to 15 mM. The samples were immediately applied onto the 4% polyacrylamide gels described in the gel mobility shift assay, and the gels were run at 150 V for 4 hr. The shift bands were visualized by imaging analyzer cut out and electroeluted. The untreated end-labeled DNA was subjected to Maxam and Gilbert (22) sequence reactions for A+G and C+T to serve as markers on the sequencing gel. The DNAs were applied onto 10% polyacrylamide gel containing 50 mM Tris-borate (pH 8.3), 1 mM EDTA and 7 M urea. The gel was run at 2500 V for 4 hr, dried and autoradiographed.

Northern blot analysis

SKT6 cells were treated with Epo (0.5 unit/ml) for 0, 0.5, 3, 48 and 72 hr, and the total cellular RNAs were prepared by the guanidine isothiocyanate/cesium chloride method (23). Poly(A)$^+$RNA (10 μg) purified by oligo(dT)-cellulose chromatography was fractionated by 1% agarose gel containing 2.2 M formaldehyde. The RNAs blotted on nitrocellulose filter were

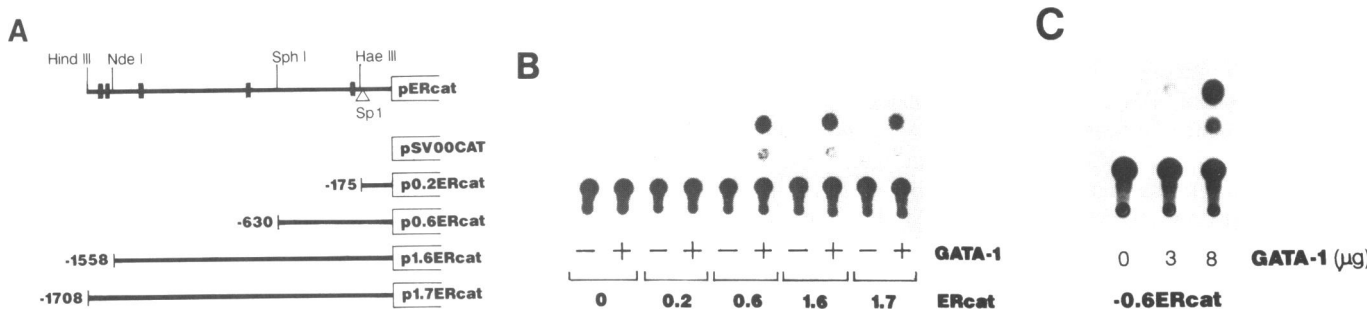

Figure 1. Transactivation of the EpoR gene expression by GATA-1. (A) Structure of the murine EpoR gene promoter and the pERcat reporter constructs. The position of several restriction sites, the Sp1 sequence (open triangle) and GATA motifs (closed boxes) are shown. The 5′ end points of pERcat deletion mutants map at positions −1708 (Hind III site), −1558 (Nde I site), −630 (Sph I site) and −175 (Hae III site), relative to the ATG codon. (B) Transient expression assay of a series of pERcat plasmids in COS 7 cells. The CAT activities in the presence (+) and in the absence (−) of pSRαGATA-1 (SRα promoter regulated mouse GATA-1 gene expression vector) are shown. pCH110 was used as internal control to normalize the transfection efficiency. (C) Dose dependent transactivation of p0.6ERcat by GATA-1. p0.6ERcat was co-transfected with 0, 3, or 8 μg of pSRαGATA-1 DNA, and CAT activity was measured.

hybridized with nick-translated GATA-1 cDNA probe. The conditions of hybridization and washing were as described (24).

RESULTS

Transactivation of EpoR gene promoter activity by GATA-1

To clarify the role of GATA motifs in EpoR promoter regions on its gene expression, plasmids containing 5′-flanking sequence of the mouse EpoR gene fused to chloramphenicol acetyltransferase (CAT) gene as a reporter were constructed (Fig.1A). Northern blot analyses showed that EpoR gene was expressed in various mouse erythroleukemia cells (SKT6, K-1 and T3Cl2−0) but not in the non-erythroid cells examined (COS 7, L and NIH 3T3)(data not shown). Thus, a plasmid containing 1.7 kb EpoR 5′-flanking regions fused to CAT gene (p1.7ERcat) along with pCH110 (coding for β-galactosidase) was co-transfected in the presence or absence of GATA-1 expression plasmid (pSRαGATA-1) into COS 7 cells. The pCH110 DNA was always co-transfected and the measured β-galactosidase activity was used to normalize the transfection efficiency.

As shown in Fig.1B, the CAT activity of p1.7ERcat was very low in the absence of GATA-1; in its presence, however, the CAT activity dramatically increased (over 10 fold)(Fig.1B). There exist five putative GATA motifs in p1.7ERcat, and to identify which of these motifs are responsible for the transcriptional activation, further analyses were performed using the deletion constructs shown in Fig.1A. The p0.6ERcat which contains a single GATA-1 binding site and anSp1 binding motif showed significant transactivation by GATA-1, and the CAT activity in the presence of GATA-1 was about the same level as p1.6ERcat and p1.7ERcat which contain 3 and 5 GATA motifs, respectively. In contrast, p0.2ERcat which contains Sp1 but not GATA-1 binding site, as well as pSV00CAT, showed no transactivation (Fig.1B). Therefore, we concluded that a single GATA motif situated at −200 to −195 bp relative to the ATG codon is sufficient to transactivate the EpoR gene by GATA-1 in COS 7 cells. The other four GATA-1 binding motifs located further upstream had no effect on the transactivation.

Figure 1C shows that CAT activity of p0.6ERcat increased in proportion to the amount of pSR GATA-1 added. Taken together, these results demonstrate that GATA-1 transactivates EpoR gene expression through a single GATA motif located around −200 bp upstream from the ATG codon in a dose dependent manner.

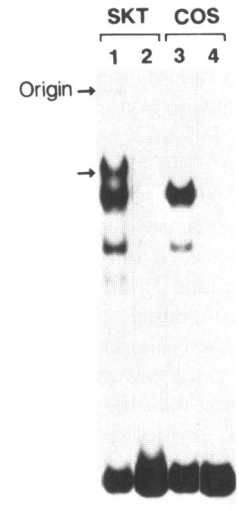

Figure 2. Erythroid-specific and ubiquitous nuclear proteins bind EpoR promoter. End-labeled 94 bp EcoT14 I-Msp I DNA fragment (−215 to −122 bp relative to the ATG codon of EpoR gene) was incubated with nuclear extracts prepared from SKT6 cells (lanes 1 and 2) and from COS 7 cells (lanes 3 and 4) in the presence (lanes 2 and 4) or in the absence (lanes 1 and 3) of the unlabeled competitor DNA, and electrophoresed.

An erythroid specific nuclear factor binds to DNA fragment containing a GATA motif

To prove definitely that the GATA-1 binding site was the essential element responsible for the GATA-1 dependent activation, a gel mobility shift assay was performed using the 94 bp DNA fragment (−215 to −122 bp relative to ATG codon) containing a single GATA-1 binding motif and an Sp1 binding site. The end-labeled 94 bp DNA fragment was incubated with nuclear extracts prepared from erythroleukemia SKT6 cells or non-erythroid COS 7 cells, and the resulting DNA-protein complexes were resolved by gel electrophoresis (Fig.2). The DNA fragment formed three major DNA-protein complexes when incubated with erythroid nuclear extracts (Fig.2, lane 1), and two with non-erythroid nuclear extracts (Fig.2, lane 3). To test the binding

specificity of the DNA-protein complexes, identical binding assays were performed in the presence of 100-fold molar excess of unlabeled competitor DNA. As shown in Fig.2, lanes 2 and 4, the unlabeled DNA completely abolished the complex formations, which indicates that all DNA-protein complexes shown in this figure are sequence-specific.

A slowly migrating complex indicated by an arrow in Fig.2 was observed only in erythroid nuclear extracts, but the other two bands are common to both erythroid and non-erythroid extracts. The latter two bands were formed not only in COS 7 but also in NIH 3T3 nuclear extracts (dat not shown). Thus, there exist at least one erythroid-specific nuclear factor and some ubiquitous nuclear factors common to erythroid and non-erythroid cells which specifically bind to the 94 bp DNA fragment spanning −215 to 0122 bp relative to ATG codon.

DNase I footprinting assay

To identify the nucleotide sequences that the erythroid-specific and ubiquitous nuclear factors bind, a DNase I footprinting assay of the 94 bp DNA fragment was performed using extracts from erythroid and non-erythroid cells (Fig.3). Analysis of the footprints revealed two binding sites for erythroid-specific and ubiquitous nuclear proteins in erythroid cells (Fig.3, lane 1). One is a region between −193 and −201 bp relative to the ATG codon where one GATA motif TTATCT is located, and the other is a region spanning −159 to −173 bp relative to the ATG codon which contains an Sp1 consensus sequence CCGCCC (Fig.3, lane 1). In contrast, there existed only one footprint with COS 7 nuclear extracts (Fig.3, lane 2), and the protected sequence was identical with the Sp1 containing region as observed in erythroid cells (Fig.3, lane 1). Exactly the same region was also protected when NIH 3T3 nuclear extracts were used (dat not shown). These results were in agreement with the gel mobility shift assay, and clearly indicate that an erythroid-specific nuclear factor is G-ATA-1 and that the ubiquitous nuclear factors common to erythroid and non-erythroid cells correspond to Sp1.

Expression of GATA-1 increases during Epo-induced erythroid differentiation

In order to examine whether mouse erythroleukemia cells expressing EpoR on their cell membrane constitutively express GATA-1, and to determine whether EpoR-mediated signals affect the GATA-1 gene expression, transcriptional levels of GATA-1 during Epo-induced erythroid cell differentiation were examined by Northern blot analysis. Mouse erythroleukemia SKT6 cells can be induce to differentiate into hemoglobin-positive cells with Epo (17, 25). After 4 days of incubation in the presence of Epo, about 75% of the cells were hemoglobin-positive, while in the absence of Epo only 3% were hemoglobin-positive. Poly(A)$^+$RNAs prepared from Epo-treated SKT6 cells for up to 72 hr were hybridized with GATA-1 probe. As shown in Fig.4, GATA-1 specific mRNA was detected in untreated SKT6 cells (lane 1), indicating that the GATA-1 gene is constitutively expressed in erythroid progenitor cells expressing EpoR. The level of the mRNA, however, increased significantly after stimulation of Epo, and after 48 hr of incubation was about 10 times the original level (lane 4), while b-actin mRNA was expressed at a constant level during Epo-induced erythroid differentiation. Thus, these results indicate that the expression of the GATA-1 gene is strongly enhanced by the EpoR-mediated signals, and that the GATA-1 transcripts increase in accordance with the appearance of hemoglobin-positive cells.

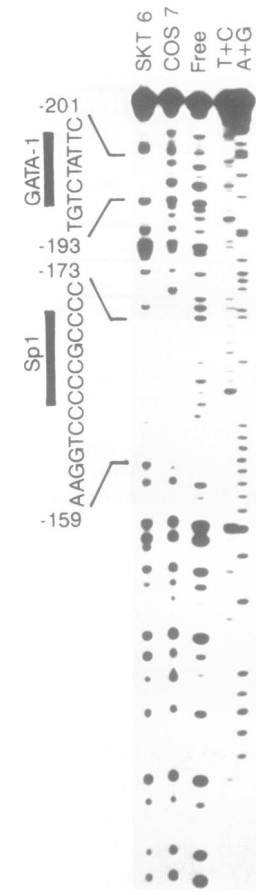

Figure 3. DNase I footprinting of 94 bp EcoT14 I-Msp I DNA fragment containing a GATA motif and an Sp1 site. The end-labeled 94 bp DNA fragment was incubated with nuclear extracts from SKT6 cells (lane 1), COS 7 cells (lane 2) or without extract (lane 3), and digested with DNase I. DNA sequencing ladders (T+C and A+G) used as markers (lanes 4 and 5).

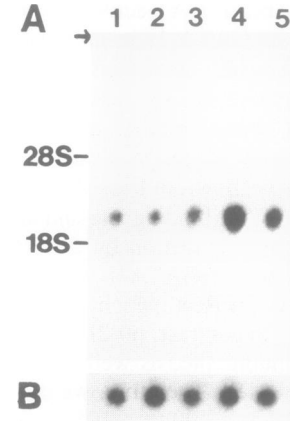

Figure 4. Analysis of GATA-1 transcripts during Epo-induced erythroid cell differentiation. SKT6 cells were harvested at the indicated times after Epo induction. (A) Poly(A)$^+$RNAs (10 μg) were fractionated by 1% agarose gel, blotted on nitrocellulose filter, and hybridized with nick-translated GATA-1 cDNA probe. Lanes 1−5, mRNAs from cells treated for 0, 0.5, 3, 48 and 72 hr, respectively. Arrow indicate the top of the gel. (B) Mouse b-actin probe was hybridized to the same blot as a control for mRNA abundance.

DISCUSSION

Interactions of Epo with specific membrane receptors on erythroid progenitor cells induce signals that are transmitted from the cell surface to the nucleus. The receptor-mediated signals are thought to involve activation of a set of specific regulatory genes that determine cell fate, i.e., proliferation or terminal differentiation. Thus, the appearance of EpoR on the cell membrane during a particular stage of eythroid differentiation is an essential event occurring during erythroid differentiation. We showed in this paper that one mechanism regulating tissue-specific and differentiation stage-specific expression of EpoR gene is transactivation of this gene by erythroid-specific transcriptional factor GATA-1. We therefore concluded that GATA-1 is expressed before EpoR appears on the cell membrane, and that EpoR (and Epo) is not a primary determinant of erythroid differentiation. GATA-1 may function as a primary determinant of erythroid lineage, and one of the most important targets of GATA-1 may be the EpoR in an early stage of erythropoiesis *in vivo*.

Transcriptional regulation of the GATA-1 gene itself then becomes a subject of considerable interest. Chromosomal structure of the gene is now under investigation and positive autoregulation is suggested because of the existence of GATA motifs in the promoter regions (unpublished data). However, the enigma of how GATA-1 gene expression is first established during the early stage of hematopoiesis remains to be resolved. In this paper, we demonstrated one of the regulatory mechanisms of GATA-1 gene expression, i.e., EpoR-mediated signals significantly amplify GATA-1 transcripts. The detailed molecular mechanism has to be resolved, but we speculate that EpoR-mediated signals might activate (e.g., by phosphorylation or dephosphorylation) GATA-1 proteins to stimulate autoregulation, or might activate the gene expression of unknown positive transcriptional factors for this gene. Taken together with other observations, these results suggest the following scheme of erythroid differentiation. Relatively low levels of GATA-1 gene products, the amounts of which are enough to transactivate the EpoR gene, are triggered to express by unknown differentiation stimuli in the early stage of hematopoiesis. Epo binds specifically to its receptor and the receptor-mediated signals enhance GATA-1 gene transcription in erythroid progenitor cells (in BFUe and CFUe). The amplified GATA-1 gene products finally transactivate hemoglobin synthesis-related genes and globin genes in relatively matured erythroid cells.

GATA-1 was first identified as a transcriptional factor activating globin gene expression upon binding to consensus GATA motif (12,13). GATA motifs are present as *cis* elements not only of globin genes but of the other known erythroid-specific genes including EpoR (3, 14, 15). These facts may indicate that the GATA-1 function is not restricted to the regulation of only globin gene but may also regulate all erythroid-specific genes.

GATA-1 related genes which possess high homology to zinc finger-like DNA binding domain have been identified in chicken, human, mouse, and xenopus (11). These nuclear factors designated GATA-2 and GATA-3 (previously known as NF-E1b and NF-E1c, respectively) are also known to bind to the same GATA motif (26). GATA-2 was expressed at a low level in various tissues through all developing stages, and GATA-3 was highly expressed in brain and T lymphocytes. GATA-binding protein which transactivates the endothelin gene was also found in endothelial cells (27). The existence of ubiquitous and various types of tissue specific GATA-binding proteins suggest the possibility that unknown GATA-binding proteins exist even in COS 7 cells and bind to GATA motifs in EpoR gene. The results of gel mobility shift assay and DNase I footprinting, however, clearly showed that only an erythroid-specific nuclear factor was found to bind specifically to the GATA motif located around −200 bp. Because of the erythroid-specific expression of GATA-1, the binding protein is likely to be GATA-1.

p0.6ERcat containing a single GATA motif was transactivated by GATA-1 in a dose-dependent manner. However, p1.7ERcat and p1.6ERcat containing 5 and 3 GATA motifs were not transactivated in proportion to their existing number of GATA motifs. Binding of GATA-1 protein to a single GATA motif may saturate the transactivation of the gene expression. Multiple GATA motifs found in many erythroid-specific genes turned out to be present in a different spacing and orientation which may affect the affinity of binding and/or biological function (11). p1.7ERcat which contains two GATA motifs in a tandem repeat only 6 bp distant showed relatively low transactivation compared with the other two constructs, which may indicate that the tandem repeat of GATA motifs located far upstream of the EpoR gene acts as a negative regulatory element. Further studies are required to clarify this point.

The EpoR gene is also known to be expressed in megakaryocytic cells (28). GATA-1 expression is also recognized in megakaryocytic and mast cell lineages (29, 30). Taken together with these facts, our current study suggested that GATA-1 plays a role in transactivating EpoR gene expression in megakaryocytic and mast cells.

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