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Developmental Silencing of Human ζ-Globin Gene Expression Is Mediated by the Transcriptional Repressor RREB1*

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The mammalian embryonic ζ-globin genes, including that of humans, are expressed at the early embryonic stage and then switched off during erythroid development. This autonomous silencing of the ζ-globin gene transcription is probably regulated by the cooperative work of various protein-DNA and protein-protein complexes formed at the ζ-globin promoter and its upstream enhancer (HS-40). We present data here indicating that a protein-binding motif, ZF2, contributes to the repression of the HS-40-regulated human ζ-promoter activity in erythroid cell lines and in transgenic mice. Combined site-directed mutagenesis and EMSA suggest that repression of the human ζ-globin promoter is mediated through binding of the zinc finger factor RREB1 to ZF2. This model is further supported by the observation that human ζ-globin gene transcription is elevated in the human erythroid K562 cell line or the primary erythroid culture upon RNA interference (RNAi)[2] knockdown of RREB1 expression. These data together suggest that RREB1 is a putative repressor for the silencing of the mammalian ζ-globin genes during erythroid development. Because ζ-globin is a powerful inhibitor of HbS polymerization, our experiments have provided a foundation for therapeutic up-regulation of ζ-globin gene expression in patients with severe hemoglobinopathies.

The human α-like (5′-ζ (embryonic)-α2-α1 (fetal/adult)-θ1 (fetal/adult)-3′) and β-like (5′-ϵ (embryonic)-^{G}γ (fetal)-^{A}γ (fetal)-δ-β (adult)-3′) globin gene clusters each extend over 50 kb on chromosomes 16 and 11, respectively. Expression of the genes within both clusters in erythroid cells are under temporal control during development (1), with reciprocal silencing of the embryonic/fetal globin genes and induction of the fetal/adult globin genes in a gene order manner (hemoglobin switch). The coordinated hemoglobin switch processes of the two clusters are also accompanied with shifting of the hematopoiesis sites. A number of previous studies have shown that the erythroid tissue- and developmental stage-specific expression of the mammalian globin gene clusters, including those of humans, are regulated by a variety of different protein-DNA and protein-protein complexes formed at different DNA sequence motifs within the globin gene promoters and their upstream regulatory elements (*i.e.* the β-LCR and α-HS-40) (2–4). These proteins include transcription factors serving as either activators or repressors, which include GATA1 (5, 6), NF-E2 (7, 8), EKLF (9, 10), YY1 (11), TR2/TR4 (12), NF-E4 (13), and BCL11A (14), etc.

Identification and detailed analysis of the transcription repressors of the embryonic/fetal globin genes would allow the design of appropriate therapeutic approaches to re-switch on these genes, thus substituting for the functioning of the defective/silenced/deleted adult α- or β-globin gene in sickle cell anemia and severe thalassemia. Previously, YY1 and GATA1 have been shown to play roles in the silencing of the embryonic human ϵ-globin gene by binding to its promoter (11, 15, 16). Similarly, evidence has been presented that the orphan nuclear receptors TR2 and TR4 silence the ϵ-globin promoter during development (12, 17). NF-E4 appeared to be a repressor of the γ-globin promoters, but its role in developmental silencing of the two γ-globin genes remains unclear (18). More recently, BCL11A has been demonstrated to be a repressor of the γ-globin gene transcription in adult erythroid cells through binding to chromosomal regions of the β-like globin gene cluster and possibly the γ-globin promoter (14, 19).

The transcriptional regulation of the embryonic ζ-globin and adult α-globin genes is controlled by the interaction between the multiple nuclear protein-DNA complexes formed on the HS-40 and the promoters (20, 21). Several erythroid-specific factor-binding sites have been identified in the 350-bp HS-40 core, including GATA1 and NF-E2, both of which are responsible for the enhancer activity of HS-40 (21, 22). Interestingly, unlike GATA1, the binding of NF-E2 to HS-40 plays not only an activation role but also negatively regulates the HS-40-mediated ζ-globin promoter activity during development. In particular, the human ζ-globin promoter is derepressed in adult transgenic mice when the 3′-NF-E2 site in the *cis*-linked HS-40 element is mutated (23). Significantly, one of the factor-binding motifs within the human ζ-promoter, as mapped by footprinting analysis (24), also plays a negative regulatory role in the activation of the ζ-globin promoter. Mutation of this motif,

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[2] The abbreviations used are: RNAi, RNA interference; hGH, human growth hormone; RT, reverse transcription; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; EMSA, electrophoretic mobility shift assay(s); siRNA, small interfering RNA; E*n*, embryonic day *n*; wt, wild type.

termed ZF2, results in an increase of the ζ-globin promoter activity in transiently transfected erythroid human K562 or mouse MEL cells (25). Moreover, this repressing role of the ZF2 motif is only apparent when HS-40 is *cis*-linked to the mutant ζ-globin promoter (25). This study has suggested that the ZF2 motif probably contributes to the autonomous silencing of the human ζ-globin promoter during development.

In the following, we show that the activity of the HS-40-linked ζ-globin promoter with the ZF2 mutated is indeed relatively higher than the wild type ζ-promoter in transgenic mice. With combined use of transient transfection, site-directed mutagenesis and electrophoretic mobility shift assays, we have also attempted to identify the putative factor(s) binding to the ZF2 motif. These assays together with RNAi knockdown experiments suggest that RREB1 is one of the factors repressing the ζ-globin promoter activity through binding to the ZF2 motif.

EXPERIMENTAL PROCEDURES

Plasmids—The construct pBS-HS40-ζ-hGH described previously was used in the current study but with replacement of the backbone with that of pBluescript II KS(−) (Stratagene) (21). This new pBS-HS40-ζ-hGH plasmid was then used as the parental plasmid to introduce different mutations into the ζ-globin promoter with the use of the QuikChange site-directed mutagenesis kit from Stratagene. pBS-HS40-α-hGH was generated by replacement of the ζ-globin promoter in pBS-HS40-ζ-hGH with a 1.5-kb PstI fragment of the α-globin promoter. pEF-Myc-RREB1 was constructed by cloning of the RREB1 cDNA (GenBank™ accession number NM_001003699) amplified by PCR using the *PfuUltra*™ II Fusion HS DNA polymerase (Stratagene), in the SalI/XhoI sites of the pEF/*myc*/cyto vector (Invitrogen).

Cell Cultures and DNA Transfection—K562 cells were maintained in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin, in a 37 °C chamber under a 5% CO_2 humidified atmosphere. MEL, HeLa, and 293T cells were cultured in the same conditions but in Dulbecco's modified Eagle's medium (Invitrogen). For DNA transfections, the cells were harvested at a density of $5–8 \times 10^5$ (K562) or $8–10 \times 10^5$ (MEL, 293T, and HeLa) per ml. The transfection was carried out using the Neon™ transfection system (Invitrogen). 2×10^6 cells were transfected with 5 μg of the test plasmids and 1 μg of pCMV-β-gal. Following microporation, the K562 cells were seeded in 6-well plates with 5 ml of antibiotic-free RPMI for 48 h before the human growth hormone (hGH) assay. The MEL cells were seeded with 5 ml of antibiotic-free Dulbecco's modified Eagle's medium for 24 h and then induced with 2% DMSO for 96 h before the hGH assay. The 293T and HeLa cells were seeded in 6-well plates with 5 ml of antibiotic-free Dulbecco's modified Eagle's medium for 48 h before the hGH assay.

Generation and Genotype Analysis of Transgenic Mice—The transgenic mice were generated in the transgenic core facility at the Institute of Molecular Biology using the standard pronuclei microinjection method. The XhoI-NotI DNA fragments isolated from the pBS-HS40-ζ-GH plasmid series (see Fig. 1*B*) were used for microinjection. For genotyping of the mouse tail DNAs, the transgene was detected with use of the primers (5′-TGCTTGTCAGGGGACAGATCC-3′ and 5′-ATTGGTCAGGTGAGGGGAGG-3′), which amplified a 464-bp product. For further structural and copy number analysis, the tail DNAs were digested with BamHI and analyzed by Southern blotting with several probes. The PA1 probe (1,026 bp) hybridized to the 5′-end of the transgene, whereas the PA3 probe (1,051 bp) hybridized to a 2.2-kb BamHI fragment. The 960-bp methyltransferase (*MT*) probe from the DNA methyltransferase I gene was used as the loading control. The XhoI-NotI fragment of the transgene was also used as the copy number standard in the Southern blotting analysis (Fig. 2*A*). After PA1 hybridization, the integrity and the single copy nature of the transgene could be determined. Use of the PA3 probe and comparison of the hybridization intensities with those of the copy number standard blot provided the copy numbers of the transgene in different lines (Table 1). Quantitation of the band intensities on the blots was carried out in a Fuji FLA-5000 phosphor imager.

TABLE 1

Copy numbers and expression levels of transgenic mice carrying the HS40-ζ-hGH transgene

The lines with the same construct are grouped at the top and bottom of the first column. The levels of hGH in the plasma collected from the individual 3-month-old mice were determined by radioimmunoassay (23). The hGH levels after normalization with the copy numbers are also listed. ND, not determined.

Line	hGH	Copy number	hGH/copy
	ng/ml		
Wild type[a]			
111	6.74	7	0.96
112	0.28	2	0.14
121	−0.32	10	ND
155	0.24	1	0.24
171	0.08	1	0.08
172	0.07	1	0.07
181	4.78	16	0.30
182	29.62	60	0.49
191	−0.31	5	ND
Non-transgenic	−0.32		
mCC[b]			
212	1.16	1	1.16
213	10.95	6	1.82
221	8.06	2	4.03
222	25.71	2	12.85
223	2.31	2	1.16
231	6.03	11	0.55
244	1.78	5	0.36
251	−0.20	4	ND
281	169.60	12	14.13
282	104.35	40	2.61

[a] The range of the hGH levels per copy of transgene in the wild type group was 0.07–1.0 ng/ml.

[b] The range of the hGH levels per copy of transgene in the mCC transgenic group was 0.3–14 ng/ml.

Semiquantitative RT-PCR Analysis—For induction of anemia, 8-month-old mice were injected with phenylhydrazine (40 μg/g of body weight), twice separated by 8 h. The treated mice were sacrificed on day 6, and the tissue RNAs were isolated by TRIzol reagent (Invitrogen). Each RT reaction was performed with the use of SuperScript II reverse transcriptase (Invitrogen) and 1 μg of RNA. One-tenth of the RT products was used as the template in PCR (Fermentas). The amplifications were carried out at the thermal cycle of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s. The products of hGH and mouse G3PDH are 313 and 525 bp, respectively. The sequences of the primers are as follows: 5′-hGH, 5′-AGGAAGGCATCCAAACGCTG-3′; 3′-hGH, 5′-ATTAGGACAAGGCTGGTGGG-3′; 5′-mouse G3PDH, 5′-GGTCATCCATGACAACTTTGG-3′; 3′-mouse G3PDH, 5′-TCTTACTCCTTGGAGGCCATG-3′.

Electrophoretic Mobility Shift Assays (EMSA)—Nuclear extracts were prepared from K562, MEL, HeLa, and 293T cells as described previously (26), in the presence of protease inhibitors (Roche Applied Science protease inhibitor mixture tablets). The oligonucleotides used are listed in Fig. 3*A*. All of the DNA binding reactions were performed as described by Wen *et al.* (27) with minor modifications. The double-stranded probes were 5′-end-labeled with ^{32}P by T4 polynucleotide kinase (New England Biolabs) and then purified over a Sephadex G-25 column (Roche Applied Science). 5 or 10 μg of the nuclear extracts were incubated with the probe (100,000 cpm) at room temperature for 15 min in 20 μl of 20 mM HEPES (pH 7.9), 50 mM KC1, 1 mM $MgCl_2$, 0.5 mM dithiothreitol, 4% glycerol, and 1 μg of poly(dI-dC). For competition EMSA, excess cold oligonucleotides were used (see legend to Fig. 3 for more details).

For further identification of the complexes by a supershift assay, the antibody anti-GATA1 (sc-265, Santa Cruz Biotechnology, Inc. (Santa Cruz, CA)) or anti-Myc (LTK BioLaboratories) was preincubated with the nuclear extract on ice for 30 min before use in EMSA. Normal IgG was used as the control.

siRNA Interference—The target sequences for siRNA interference of RREB1 mRNA (siRNA 1, 5′-GGGCAGACCUUUCAUACAGUU-3′; siRNA 2, 5′-GAAGAAAGCUGAUGAAGUCUU-3′) were identified using the manufacturer's design (ON-TARGETplus, Dharmacon). One strand of the control duplex RNA targeting the firefly luciferase mRNA is 5′-CUUACGCUGAGUACUUCGAUU-3′. 2×10^6 K562 cells were transfected with the duplex RNA oligonucleotides at a concentration of 100 nM. Using the Neon™ transfection system (Invitrogen), cells were microporated at 1,300 V with a 30-ms width and one pulse and then reseeded in 2 ml of antibiotic-free RPMI. 2 ml more of the medium were added to the cells 6 h later. After incubation for 48 h, half of the cells were harvested for assay of the gene expression. The remnants were remicroporated with siRNA oligonucleotides using the same conditions and incubated for another 48 h before the assay.

Lentivirus-mediated Knockdown Experiments—Lentiviral plasmids (pLKO.1-shRNA) expressing short hairpin RNAs (shRNA1, 5′-CCGGCCAGGAAACGAAAGAGGAGAACUCGAGUUCUCCUCUUUCGUUUCCUGGUUUUU-3′; shRNA2, 5′-CCGGCGACGAUGACAAGAAACCAAACUCGAGUUUGGUUUCUUGUCAUCGUCGUUUUU-3′) targeting the RREB1 mRNA were acquired from the TRC (The RNAi Consortium) lentiviral shRNA library (28). pLKO.1sh expressing a scrambled shRNA and the shRNA-null puromycin-resistant vector (pLKO.1) were used to produce the control lentiviruses. The viruses were prepared by co-transfecting 293T cells with the plasmid (pLKO.1-shRNA, pLKO.1sh, or pLKO.1), the packaging plasmid (pCMV-ΔR8.91), and the envelope plasmid (pMD.G). The culture medium containing lentiviruses was harvested at 64 h post-transfection for the estimation of the viral titer. For RNAi knockdown in K562 cells, spin infection (multiplicity of infection = 2) was carried out at 2,750 g in 6-well plates for 30 min at 25 °C, with a final concentration of 8 μg/ml polybrene in the culture medium. After 24 h of lentiviral infection, the cells were selected with 2.5 μg/ml puromycin for another 4 days. The total RNAs were harvested from cells at 5 days and 10 days postinfection, respectively, for further quantitative RT-PCR analysis.

For RNAi knockdown experiments of primary human erythroid culture, the culture was initiated and prepared following the standard protocol (29) except that StemSpan SFEM medium (StemCell Technologies) was used for culturing and maintenance of the cells (14). The cells were maintained in the differentiation medium at a density of $0.1–1 \times 10^6$ cells/ml. The lentivirus transductions were carried out on day 2 of the phase II culture of erythroid differentiation. Puromycin (2 μg/ml) selection was started at 24 h post-transduction for 9 days, and the total RNAs were then isolated using the RNAqueous®-Micro Kit (Ambion) for analysis by quantitative RT-PCR.

Quantitative RT-PCR Analysis—RNAs from E9.5 mouse embryos with the yolk sac and the E14.5 mouse fetal liver were isolated with use of TRIzol reagent (Invitrogen). The RNAs of the RNAi knockdown cells were purified using the RNAspin mini kit (GE Healthcare). cDNA synthesis was carried out using SuperScript II reverse transcriptase (Invitrogen). Quantitative RT-PCR was performed by using the SYBR Green PCR Master Mix (Applied Biosystems) and ABI 7500 real-time system. All data were analyzed after normalization to the expression level of mouse glycophorin A or human β-actin gene. The sequences of the primers used for the quantitative RT-PCR are as follows: 5′-hGH, 5′-TAGAGGAAGGCATCCAAACG-3′; 3′-hGH, 5′-GTCTGCTTGAAGATCTGCCC-3′; 5′-mouse GPA, 5′-GCCGAATGACAAAGAAAAGTTCA-3′; 3′-mouse GPA, 5′-TCAATAGAACTCAAAGGCACACTGT-3′; 5′-human β-actin, 5′-CCTGAACCCCAAGGCCAACC-3′; 3′-human β-actin, 5′-CAGGGATAGCACAGCCTGGA-3′; 5′-RREB1, 5′-CGACTTAGGATTCACGGACTTC-3′; 3′-RREB1, 5′-CAGACAAAACGGTGTTGCTC-3′; 5′-human GATA1, 5′-TGGCCTACTACAGGGACGCT-3′; 3′-human GATA1, 5′-CATATGGTGAGCCCCCTGG-3′; 5′-human G3PDH, 5′-CAACTTTGGTATCGTGGAAGGACTC-3′; 3′-human G3PDH, 5′-AGGGATGATGTTCTGGAGAGCC-3′.

RESULTS

Functional Role of the ZF2 Motif in Erythroid Cell Cultures—The factor-binding motifs in the human ζ-globin promoter region from −250 to −70, as determining previously by footprinting analysis in K562 nuclear extract (24), are displayed in Fig. 1*A*. Among these factor-binding motifs, ZF2 (−169 to −148) (Fig. 1*A*) consists of a GATA-1 binding site followed by a sequence homologous to the consensus of the binding sites of the factor RREB1 (Fig. 1*B*). To examine the contributions of the putative GATA-1 and RREB1 binding sites within the ZF2 motif to the ζ-globin promoter activity, we introduced three different types of mutations (mT, mCC, and 3nt; Fig. 1*B*) into the ZF2 motif of the promoter. The activities of the wild type ζ-promoter and the three mutants were then compared by hGH reporter assay in transiently transfected K562 and MEL cells (Fig. 1*B*). As seen, the abolishment of the GATA-1 binding site on ZF2 (mutant mT) caused significant reduction of the ζ-promoter activity in K562 as well as in MEL cells (Fig. 1*B*, *gray bars*). On the other hand, mutation of the putative RREB1 binding site (mCC) resulted in ~2-fold higher promoter activity in either K562 or MEL cells (Fig. 1*B*, *black bars*) but not in the non-erythroid 293T and HeLa cells in which the ζ-globin promoter activities were very low (Table 2). The elevated activity of

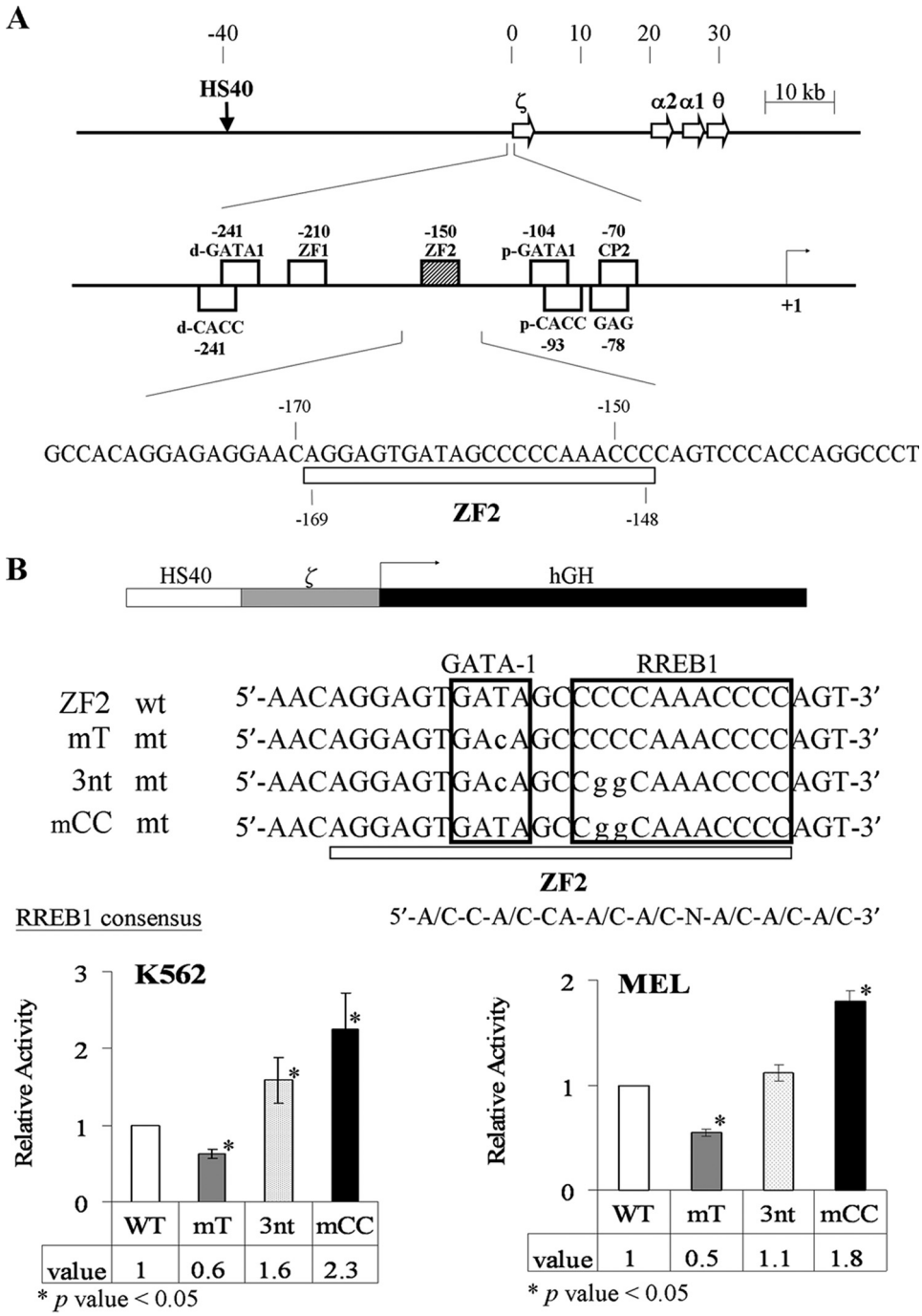

FIGURE 1. **Assessment of ζ-globin promoter activity in erythroid cell cultures.** *A*, schematic illustrations of the α-like globin locus and the ζ-globin promoter region. The physical maps of the α-like globin gene cluster and the protein binding sites in the ζ-globin promoter are shown. Shown *below* the ζ-globin promoter is the ZF2 motif. The *striped bar* indicates the position of the ZF2 motif as mapped previously by footprinting analysis (24). *B*, the promoter-reporter construct is represented in the *upper diagram*. The reporter is hGH as driven by the human ζ-globin promoter (ζ) *cis*-linked with the HS-40 enhancer. The *lowercase letters* represent the mutated nucleotides in ZF2. The consensus GATA-1 and RREB1 sequences are *boxed*. Also listed is the consensus sequence of RREB1 binding sites (31). At 48 or 96 h after transfection, the culture media were collected, and the hGH levels were determined by radioimmunoassay. *, $p < 0.05$. The data were derived from three independent experiments.

the mCC mutant promoter in the erythroid cell lines was consistent with the previous study (25), further suggesting that a factor(s) binding to the predicted RREB1 site of ZF2, possibly RREB1, was a repressor of the human ζ-globin promoter.

Physiological Role of ZF2 Motif in Transgenic Mice—To further address the physiological role of ZF2 in the regulation of the ζ-globin promoter, we analyzed transgenic mice carrying the same ζ-GH reporter fragments as used in Fig. 1, with or without the mCC mutation (Table 1 and Fig. 2). The positive lines were first identified by the presence of a 464-bp PCR fragment amplified from the human ζ-promoter region (see "Experimental Procedures") (data not shown). These lines were further analyzed by Southern blotting, as exemplified in Fig. 2*A*. The copy numbers of the transgene were determined and listed in Table 1. They varied from 1 to 60 for the wild type transgene and from 1 to 40 for the mutant transgene (Table 1). As measured by the hGH assay of the plasma samples from the adult transgenic mice, the activities of the wild type human ζ-globin promoter in most of the transgenic lines were very low, as also observed previously (23). In contrast to the wild type, the mutant lines, except for line 251, showed a significantly higher level of hGH per copy of the transgene. In particular, for the low copy lines (*e.g.* 221, 222, and 223), the levels of the plasma hGH per copy of the mutant transgene were 10–40 times higher than in mice carrying the wild type promoter (Table 1).

We have also analyzed the hGH expression in the mutant lines by RT-PCR analysis. First, to analyze the level in the adult mice, the mice were treated with phenylhydrazine to increase the erythropoiesis. RNAs were then isolated from the blood, spleen, liver, kidney, and brain and analyzed by semiquantitative RT-PCR. As shown in Fig. 2*B*, for both the wild type and the mutant transgenic lines, the RT-PCR signals were detected mainly in the blood samples (Fig. 2*B*, *lanes B*), although a minor signal could also be seen in the spleen samples (Fig. 2*B*, *lanes S*). Finally, quantitative RT-PCR analysis showed that the mutant ζ-globin promoter was also derepressed in the yolk sac of E9.5 embryos and fetal liver of E14.5 embryos (Fig. 2*C*). The data of Fig. 2 suggested that ZF2 also played a repressive role in the regulation of the human ζ-globin promoter activity *in vivo* during erythroid development.

Factor Binding at the ZF2 Motif—Following the above, we have used EMSA to examine the nature of the complex(es) formed on the ZF2 motif, in particular at the RREB1 sequence. For this, four oligonucleotides containing the wild type and the three different mutant ZF2 sequences listed in Fig. 1*B* were used as the EMSA probes (Fig. 3*A*). As shown in *lanes 2–5* of Fig. 3*B*, four major complexes (*a*, *b*, *G*, and *?*) were present on the EMSA gels when the wild type (*wt*) ZF2 oligonucleotide was used. Of the four, the *band* marked with a *question mark* was present only in K562 nuclear extract, and it formed with all four oligonucleotides (Fig. 3*B*). This complex band was not studied further. Of the other three, complex a formed in all four nuclear extracts tested, whereas complex G appeared only in the erythroid extracts (compare *lanes 2* and *3* with *lanes 4* and *5*; Fig. 3*B*). Complex b was not present in the uninduced MEL extract (Fig. 3*B*, *lane 3*).

As described in Fig. 1 already, ZF2 consisted of two factor-binding sites, one for GATA-1 and the other homologous to the RREB1 consensus binding sequence. To correlate the three EMSA complexes (complexes a, b, and G) with these factors, oligonucleotides containing mutations at the GATA-1 site (mT), the RREB1 sequence (mCC), and both (3nt), respectively (Fig. 3*A*), were used in EMSA. As shown, when oligonucleotide ZF2 (mCC) was used, mainly band a disappeared (Fig. 3*B*, compare *lanes 7* and *8* with *lanes 2* and *3*), suggesting that band a was a protein-DNA complex formed at the RREB1 sequence. On the other hand, band G disappeared when oligonucleotide ZF2 (mT) was used (Fig. 3*B*, compare *lanes 10* and *11* with *lanes 2* and *3*), suggesting that it was a GATA-1-DNA complex. At the same time, the amounts of both complexes a and b increased in EMSA with the mT oligonucleotide (Fig. 3*B*, *lanes 10* and *11*). We interpreted the data of Fig. 3*B* as partly the result of the close proximity and possibly the overlapping nature of the RREB1 sequence and the GATA-1 binding site (see sequence of the oligonucleotide ZF2 (*wt*) in Fig. 3*A*). When GATA-1 binding site was mutated, more bindings of factors (*e.g.* RREB1) to the RREB1 sequence and other yet-to-be-determined factor-binding sites on the oligonucleotide became possible, thus forming more complexes a and b, respectively. In agreement with the EMSA data above, on the gel with use of the ZF2 (3nt) oligonucleotide, in which both the GATA-1 binding site and RREB1 sequence were mutated, complex a as well as complex G disappeared, whereas complex b increased significantly (Fig. 3*B*, *lanes 13* and *14*).

The above suggested factor-binding scenario at the ZF2 motif in the K562 nuclear extracts was further confirmed by EMSA with ^{32}P-labeled probe(s), a 50–200-fold molar

TABLE 2

Expression levels of hGH in transfected cells

5 μg of the two hGH-expressing reporter plasmids were transfected into different types of cells. The amounts (ng/ml) of hGH in the cell medium at 48 h post-transfection were measured and normalized with the β-galactosidase activity from the co-transfected plasmid pCMV-β−gal.

Cell type	pBS-HS40-ζ-hGH				pBS-HS40-α-hGH (wild type)
	Wild type	mT	3nt	mCC	
	ng/ml				*ng/ml*
K562	999	617	1,486	2,096	6,104
MEL	390	215	440	697	2,673
293T	9.9	13.8	14.1	10.0	
HeLa	3.9	10.6	8.5	3.4	

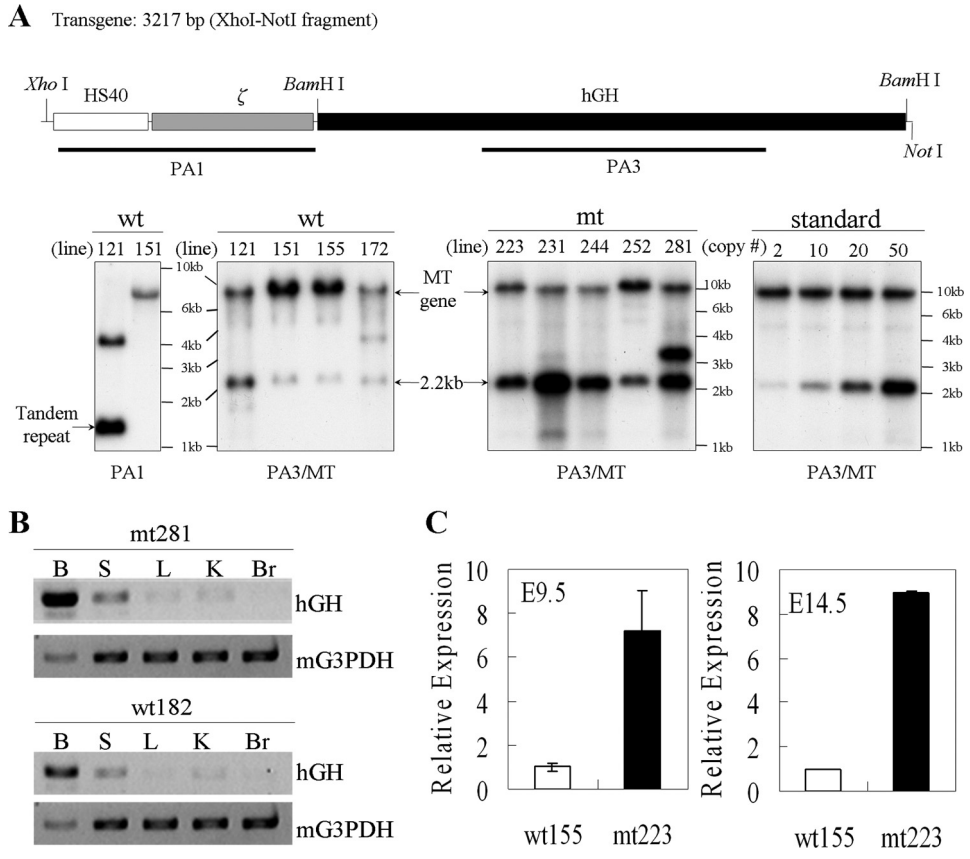

FIGURE 2. **Analysis of transgenic mice.** *A*, generation of the transgenic mice. *Top*, the diagram of the XhoI-NotI DNA fragment used for generation of the transgenic mice. The probes (PA1 and PA3) used for genotyping by Southern blotting are indicated by *underlines under* the reporter map. The copy numbers of the transgene were determined by Southern blotting as exemplified in the *lower panels*. The genomic DNAs from the tails were digested with BamHI and then hybridized with the probes. The DNA sizes of the markers are indicated on the *right sides* of the blots. The position of the head-to-tail tandem repeats of the transgene are marked on the *side* of the *left blot*. The endogenous DNA methyltransferase gene (*MT*) serves as the loading control on the blots. For copy number determination of the transgene, known copies of BamHI-digested XhoI-NotI fragment were loaded on gel and probed with PA3 (*right*). *B*, tissue-specific expression patterns of the wild type (*wt*) and mCC (*mt*) ζ-globin promoters in transgenic mice. The phenylhydrazine-treated, anemic mice were sacrificed, and the total RNAs were purified from several different adult tissues. The levels of the hGH RNAs were determined by semiquantitative RT-PCR using mouse G3PDH as the internal control. *B*, blood; *S*, spleen; *L*, liver; *K*, kidney; *Br*, brain. *C*, quantitative RT-PCR analysis of hGH mRNA in total RNAs isolated from E9.5 embryos and E14.5 fetal livers. Note the higher hGH mRNA levels in samples with mutant human ζ-globin promoter transgenes. *wt*, wild type.

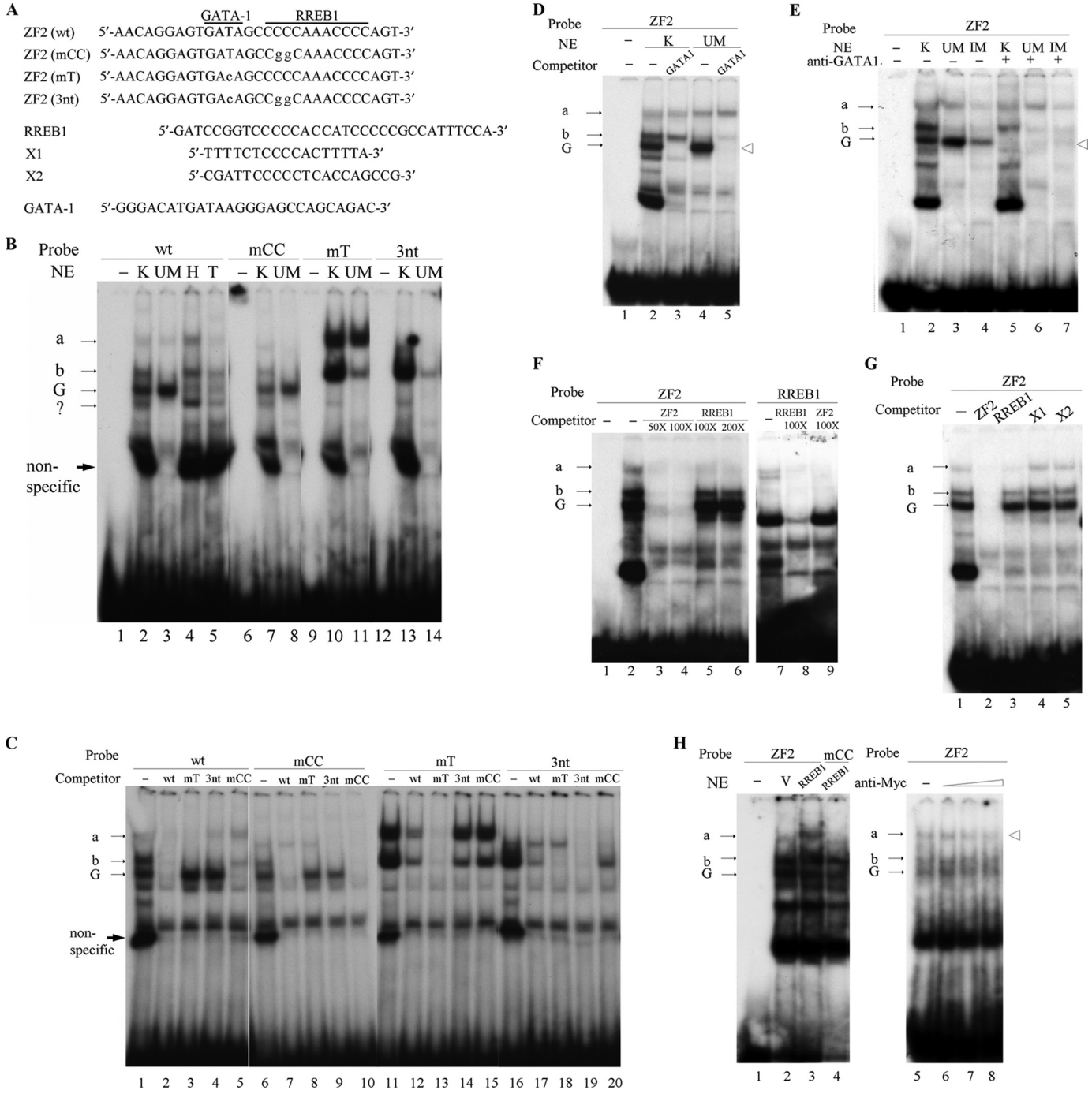

FIGURE 3. **EMSA of factor-binding on the ZF2 motif.** The identification of factors binding to the ZF2 motif in nuclear extract was analyzed by EMSA. *A*, nucleotide sequences of the oligonucleotides used for EMSA. Only one strand of each oligonucleotide is shown. The GATA-1 and RREB1-binding sequences on the wild type ZF2 are indicated. *B*, formation of DNA-protein complexes in nuclear extracts prepared from K562 (*K*), uninduced MEL (*UM*), HeLa (*H*), and 293T (*T*) cells. The four slowly migrating DNA-protein complexes formed in the K562 (*K*) extracts (*a*, *b*, *G*, and ?) are indicated. Note the presence of complex G only in the *K* and *UM lanes*. Also, the complex a is absent when the ZF2 (*mCC*) or ZF2 (*3nt*) oligonucleotide was used as the probe. *C*, competition among different ZF2 oligonucleotides. The factor-binding specificities on the ZF2 motif in K562 nuclear extract were determined with or without the presence of a 100-fold molar excess of unlabeled oligonucleotides, as indicated in the figure as the competitors. *D*, competition between ZF2 and GATA-1 oligonucleotides in EMSA. Both the K562 and uninduced MEL extracts were used. Note that complex G but not complex a disappeared (*arrowhead*) in the presence of a 100-fold molar excess of cold GATA-1 oligonucleotide. *E*, supershift assay using anti-GATA1. Nuclear extracts from three different cell types were prepared as described above, preincubated with the anti-GATA1 antibody, and then used in EMSA. Note the disappearance (*arrowhead*) of *band G* but not *band a* or *band b* upon preincubation with anti-GATA1 (*lanes 5–7*). *F*, competition between ZF2 and RREB1 oligonucleotides in EMSA. Note that complex a but not complex G or b formed on the ZF2 (*wt*) oligonucleotide disappeared upon use of a 100–200-fold molar excess of cold RREB1 oligonucleotide (*lanes 5* and *6*). All three complexes disappeared in the presence of cold ZF2 (*wt*) oligonucleotide (*lanes 3* and *4*). *G*, competition among ZF2, RREB1, X1, and X2 oligonucleotides. Note that complex a formed on the ZF2 oligonucleotide was competed out by an excess of cold ZF2 (*lane 2*) or RREB1 oligonucleotide (*lane 3*) but not by a 100-fold molar excess of X1 (*lane 4*) or X2 (*lane 5*). *H*, supershift assay using anti-Myc. *Left*, EMSA patterns using the ZF2 (*wt*) oligonucleotide (*lanes 1–3*) or ZF2 (*mCC*) oligonucleotide (*lane 4*) and nuclear extracts prepared from K562 cells transfected with pEF-Myc vector (*lane 2*) and pEF-Myc-RREB1 (*lanes 3* and *4*), respectively. Note the increase of the complex *band a* in *lane 3*. *Right*, patterns of EMSA using the ZF2 oligonucleotide and Myc-RREB1-overexpressing K562 nuclear extract without (*lane 5*) or with preincubation with increasing amounts of the anti-Myc antibody (*lanes 6–8*). *wt*, wild type.

excess of cold oligonucleotide competitors, and supershift assays (Fig. 3, *C–H*). For example, the formation of complex G with the ZF2 (*wt*) oligonucleotide as the probe was abolished when a 100-fold excess of cold, GATA-1-binding site-containing oligonucleotides (*i.e.* ZF2 (*wt*) (Fig. 3*C*, *lane 2*), ZF2 (mCC) (Fig. 3*C*, *lane 5*), and GATA-1 (Fig. 3*D*, *lanes 3* and *5*)) (30) were preincubated with the nuclear extracts. That complex G, a GATA-1-DNA complex, was also confirmed with the use of an anti-GATA-1 antibody in a supershift assay (Fig. 3*E*). Similarly, the formation of the complex a was competed away with the use of the ZF2 (*wt*) or ZF2 (mT) oligonucleotide as the competitor (Fig. 3*C*, *lanes 2* and *3*) but not by the ZF2 (3nt) or ZF2 (mCC) oligonucleotide (Fig. 3*C*, *lanes 4* and *5*). More significantly, the formation of complex a on the wild type ZF2 oligonucleotide in the K562 nuclear extract could be effectively competed by an excess of either cold ZF2 oligonucleotide itself or by cold RREB1-binding site-containing oligonucleotide (Fig. 3*F*, *left*), the latter of which was shown before to bind the RREB1 factor specifically (31). The reverse competitive EMSA experiment gave a similar result (Fig. 3*F*, *right*). Furthermore, the use of the oligonucleotide X1 or X2, both of which consisted of sequences unrelated to the RREB1-binding consensus, as the competitor could not abolish the formation of complex a, whereas the ZF2 and RREB1 oligonucleotides could (Fig. 3*G*, compare *lanes 4* and *5* with *lane 3*).

That band a was a RREB1-DNA complex was further supported by a supershift assay. Because no anti-RREB1 of supershift quality was available, we overexpressed Myc-tagged RREB1 by transient transfection of K562 cells with the plasmid pEF-Myc-RREB1 (Fig. 3*H*, *left*, *lane 3*). As seen in the *right panel* of Fig. 3*H*, preincubation of the Myc-RREB1-containing nuclear extract with anti-Myc resulted in the decrease of the intensity of band a (Fig. 3*H*, compare *lanes 7* and *8* with *lanes 5* and *6*). The data of Fig. 3 together strongly suggested that RREB1 was the factor binding to the RREB1 sequence in the ZF2 motif.

Elevated Expression of the Human ζ-Globin Gene in RREB1-depleted Cells—To further verify the negative regulatory effect of RREB1 on the ζ-globin promoter activity, as suggested above by data from Figs. 1–3, we first knocked down the RREB1 expression in K562 cells by RNAi. For this, two different siRNA oligonucleotides were used, both of which were targeted at two specific sequences on the human RREB1 mRNA. After the RNAi treatment of K562 cells, the RNA was isolated and analyzed by quantitative RT-PCR. Of the two siRNA oligonucleotides, siRNA 1 reduced the level of RREB1 mRNA by 67%, and siRNA 2 reduced the level by 35% after 48 h of treatment. A small increase of the ζ-globin mRNA (1.4-fold) was observed. To our surprise, reduction of RREB1 also caused an increase in the level of the α-globin mRNA by ~1.7-fold, whereas those of the GATA-1 mRNA and G3PDH mRNA were not altered (Fig. 4*A*, *top*). At 96 h of the siRNA treatment, more increases of the two globin mRNAs were observed, with the ζ-globin mRNA elevated by 2-fold and the α-globin mRNA elevated by 2.7-fold in siRNA 1-treated samples (Fig. 4*A*, *bottom*).

We also analyzed the level of the ζ-globin mRNA in K562 cells after more persistent reduction of the RREB1 expression with the use of two different recombinant lentiviruses, each

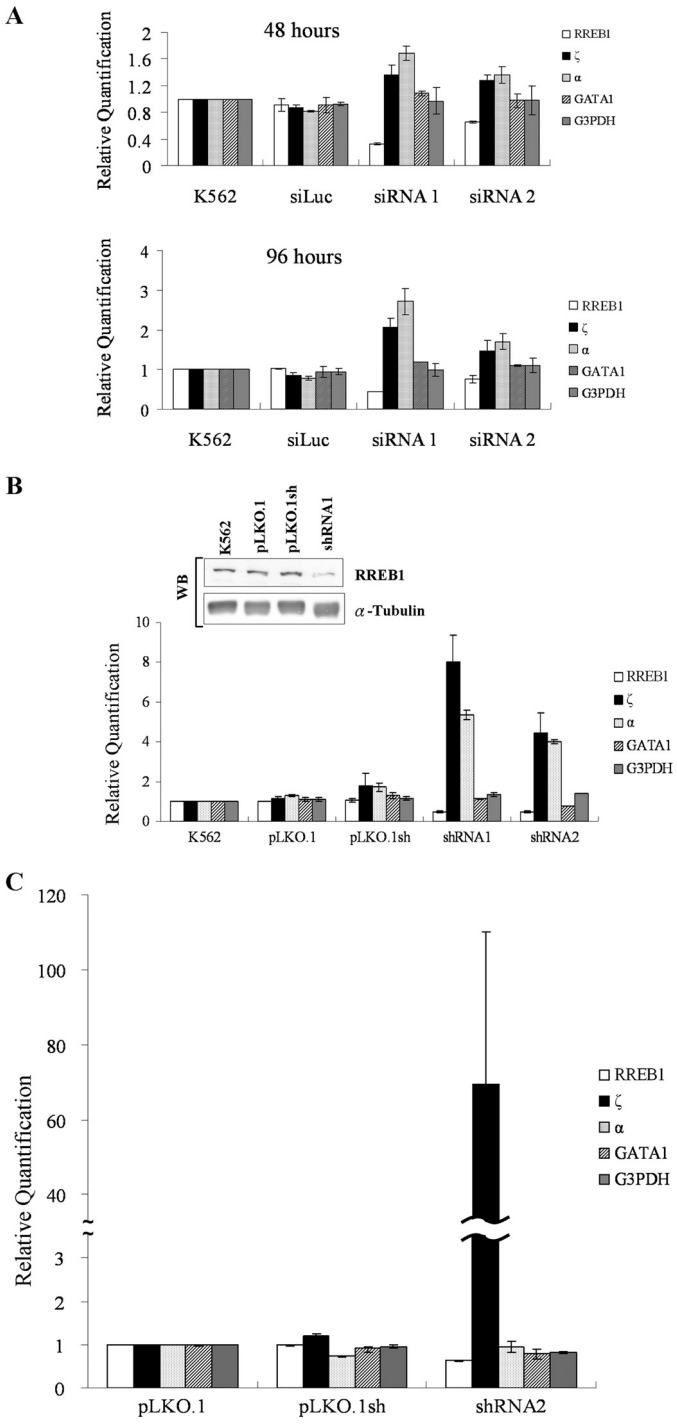

FIGURE 4. **The expressional levels of α-like globin genes in RREB1-depleted cells.** *A*, siRNA oligonucleotides were transiently transfected into K562 cells by electroporation. The cells were collected 48 h later to purify the RNA for analysis. The remnants were re-electroporated with the same siRNA oligonucleotides again. The *upper histogram* represents the data after 48 h of transfection. The *bottom panel* consists of data at 96 h post-transfection after the two sequential transfections of the RNAi oligonucleotides. A luciferase siRNA was used as the nonspecific control. Two independent siRNA oligonucleotides targeted to the RREB1 mRNA were used. *B*, lentivirus-mediated knockdown of RREB1 mRNA in K562 cells. Cells were infected with the indicated lentiviruses and then collected on the 10th day after viral infection for RNA analysis by quantitative RT-PCR. The *panel* shows the level of the RREB1 protein, as analyzed by Western blot (*WB*). *C*, lentivirus-mediated knockdown of RREB1 mRNA in primary human erythroid cells. Primary cultures of human erythroid cells were infected with lentivirus carrying shRNA2 targeting the RREB1 mRNA as described under "Experimental Procedures." The total RNAs were isolated at the 10th day postinfection and subjected to quantitative RT-PCR analysis.

expressing an shRNA targeting the RREB1 mRNA. The empty lentiviral vector was used as the control. As seen, the RREB1-shRNAs reduced the RREB1 mRNA levels to 40% after 5 days of infection (data not shown), and the knockdown of RREB1 mRNA could last for 10 days (Fig. 4*B*). Western blot using an anti-RREB1 antibody from Rockland indicated that the RREB1 protein level was indeed lowered after knockdown of the RREB1 mRNA (Fig. 4*B*, *inset*). The ζ-globin mRNA also remained at a high level after 10 days of infection, ~4–8-fold higher than the controls (Fig. 4*B*). Notably, the increases of the ζ- and α-globin gene expression, as shown in Fig. 4, were not by an indirect effect due to the induction of erythroid differentiation because the mRNA levels of neither erythroid-related genes (*e.g.* GATA-1 and NF-E2) nor non-erythroid genes (*e.g.* G3PDH) were significantly different between K562 cells with and without depletion of the RREB1 mRNA by RNAi knockdown (Fig. 4) (data not shown).

Finally, we have tested the effect of knockdown of RREB1 expression by lentivirus-based shRNA in primary culture of adult human erythroid cells. Although shRNA1 could not effectively knock down the level of RREB1 mRNA in the primary culture (data not shown), expression of the lentivirus-based shRNA2 consistently reduced the RREB1 mRNA level by 50% (Fig. 4*C*). Interestingly, this reduction greatly increased the level of the ζ-globin mRNA, by 70-fold, but not that of the α-globin mRNA (Fig. 4*C*). As in RNAi-knockdown K562 cells, the levels of the GATA1 and G3PDH mRNAs were not altered either (Fig. 4*C*). These data of Fig. 4 strongly suggested that the RREB1 played a repressive role in the human ζ-globin gene expression *in vivo*.

DISCUSSION

In this study, we have explored the possibility of re-switching on the human embryonic ζ-globin gene at the adult stage by manipulating the formation of protein-DNA complex at a sequence motif, ZF2, in the ζ-globin promoter region. We have also explored the identity of the factor(s) bound at ZF2 and repressing the ζ-globin promoter activity. Our data suggest that binding of the factor RREB1 at ZF2 participates in the negative regulation of the ζ-globin gene transcription during erythroid development.

Initially, the repressive role of ZF2 has been revealed from previous studies of mutagenized ζ-globin promoter in K562 and MEL (25), both of which are well established erythroid cell lines (32–37). The globin promoter activities from the different reporter plasmids were consistent with the cell type and developmental stage specificities of globin gene expression in the cell lines transfected. For example, the ζ-globin promoter activity was lower in MEL than in K562, and it is extremely low in non-erythroid 293T and HeLa cells (Table 2). The role of ZF2 in K562 and MEL has been confirmed in the present study (Fig. 1). Although mutation of the GATA-1 binding site in ZF2 decreases the ζ-promoter activity, suggesting that GATA-1 is an activator, the mCC mutation of the adjacent RREB1 sequence derepresses the ζ-globin promoter in erythroid cell lines (Fig. 1*B*) and in erythroid cells of the transgenic mice (Fig. 2). For the latter, the hGH levels in the blood samples of the transgenic mice carrying the wild type HS40-ζ-hGH construct are either very low or undetectable, except for line 182 (Table 1). The high copy number of the tandemly arranged transgenes in this line may have generated a novel transcription milieu that partially overcomes the silencing effect from the surrounding chromatin environment. On the other hand, the erythroid-specific activities of the mCC mutation-carrying human ζ-globin promoter in different transgenic mouse lines at the adult stage are mostly 10–40-fold higher than those of the wild type ζ-globin promoter (Table 1). The mutant promoter activity is also 7–10-fold higher than that of the wild type in E9.5 and E14.5 embryos (Fig. 2*C*). Note that the ζ → α hemoglobin switch already has occurred at E7.5, the earliest stage of erythroid development with manipulatable samples for experimentation (38–41).

The results from the DNA transfection studies in erythroid cell lines and transgenic mouse analysis suggest that factor binding at the RREB1 sequence in the ZF2 motif plays a key role in the silencing of the ζ-globin promoter during erythroid development. RREB1 is a ubiquitously expressed, ~180-kDa zinc finger protein (31) that represses several other promoters (*e.g.* p16 and PSA) through binding to the RREB1 sites in these promoters (42, 43). Furthermore, the repression by RREB1 is probably mediated through the RREB1-containing CtBP co-repressor complex (44). Although we have not been able to carry out chromatin-immunoprecipitation experiments due to the inaccessibility of appropriate anti-RREB1 antibody, several lines of evidence from our studies are highly suggestive that RREB1 is a factor, if not the only one, involved in the repression of the human ζ-globin gene *in vivo* through direct binding at the ZF2 motif. First, the RREB1 sequence of ZF2 (Fig. 1*B*) is highly homologous to the binding consensus of RREB1, 5′-(A/C)C(A/C)CA(A/C)(A/C)N(A/C)(A/C)(A/C)-3′ (Fig. 1*B*) (31). Second, the mCC mutation at the CC dinucleotides (which are conserved among all known and well characterized RREB1 binding sites (31)) of the RREB1 sequence on ZF2 abolishes its binding by the RREB1 factor, as suggested by the EMSA data (Fig. 3). At the same time, the same C → G substitutions lead to the derepression of the ζ-globin promoter activity in erythroid cell lines and in the erythroid cells of transgenic mice (Figs. 1*B* and 2). Finally, RNAi knockdown of the RREB1 level could elevate the mRNA level of the ζ-globin gene in K562 cells as well as in primary culture of adult human erythroid cells (Fig. 4). With respect to the last result, it is interesting to note first that the α-globin mRNA in the embryonic/fetal erythroid cell line K562 is also elevated upon knockdown of RREB1 and that there also exists an RREB1-binding site-like sequence (5′-GCCCCCAG-CCCAGCCCCGT-3′) in the α-globin promoter at −674 to −661. More remarkably, RNAi knockdown of RREB1 expression in the adult human erythroid culture significantly elevates the level of the ζ-globin mRNA but not the α-globin mRNA. This suggests that RREB1 is involved in the silencing of the mammalian embryonic ζ-globin promoter during the embryonic/fetal to adult erythroid development and, reciprocally, the repression of the α-globin promoter at the early embryonic/fetal stages. Interestingly, RREB1 also behaved as a repressor of ϵ-globin gene transcription.[3]

[3] R.-L. Chen and C.-K. J. Shen, unpublished results.

In summary, the data described in this study identify RREB1 as a repressor involved in the developmental silencing of the human ζ-globin gene and probably that of other mammals as well. The repression of the embryonic ζ-globin gene by RREB1 is in interesting analogy to the other two autonomously regulated human globin genes (*i.e.* the embryonic ϵ-globin gene by YY1 (11) and TR2-TR4 (12) and the fetal γ-globin gene by NF-E4 (18) and BCL11A (14)). The identification of RREB1 as a possible switch factor for the ζ-globin gene expression provides a new research target for the treatment of certain forms of severe α-thalassemia.

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