Specific repression of β-globin promoter activity by nuclear ferritin

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Developmental hemoglobin switching involves sequential globin gene activations and repressions that are incompletely understood. Earlier observations, described herein, led us to hypothesize that nuclear ferritin is a repressor of the adult β-globin gene in embryonic erythroid cells. Our data show that a ferritin-family protein in K562 cell nuclear extracts binds specifically to a highly conserved CAGTGC motif in the β-globin promoter at −153 to −148 bp from the cap site, and mutation of the CAGTGC motif reduces binding 20-fold in competition gel-shift assays. Purified human ferritin that is enriched in ferritin-H chains also binds the CAGTGC promoter segment. Expression clones of ferritin-H markedly repress β-globin promoter-driven reporter gene expression in cotransfected CV-1 cells in which the β-promoter has been stimulated with the transcription activator erythroid Krüppel-like factor (EKLF). We have constructed chloramphenicol acetyltransferase reporter plasmids containing either a wild-type or mutant β-globin promoter for the −150 CAGTGC motif and have compared the constructs for susceptibility to repression by ferritin-H in cotransfection assays. We find that stimulation by cotransfected EKLF is retained with the mutant promoter, whereas repression by ferritin-H is lost. Thus, mutation of the −150 CAGTGC motif not only markedly reduces *in vitro* binding of nuclear ferritin but also abrogates the ability of expressed ferritin-H to repress this promoter in our cell transfection assay, providing a strong link between DNA binding and function, and strong support for our proposal that nuclear ferritin-H is a repressor of the human β-globin gene. Such a repressor could be helpful in treating sickle cell and other genetic diseases.

During development of vertebrates, genes of the globin family are differentially regulated to produce different combinations of peptides that assemble into hemoglobin (Hb) tetramers with different physiological properties, a phenomenon termed developmental Hb switching (1–3). The human α-like and β-like globin genes, arrayed in separate clusters on chromosomes 16 and 11, respectively, are coordinately regulated to produce first, embryonic Hb (e.g., $\zeta_2\varepsilon_2$), followed by fetal Hb (e.g., $\alpha_2^A\gamma_2$), and then, adult Hb (e.g., $\alpha_2\beta_2$).

Previous studies have shown that developmental regulation of globin genes is complex, involving chromatin remodeling (4) as well as interactions among multiple trans-acting factors (1, 3). These include GATA-1, NF-E2, SSP/NF-E4, erythroid Krüppel-like factor (EKLF), and other proteins (2, 5–9). For example, EKLF, a positive regulator specific for the adult β-globin promoter (8), requires posttranslational modification and/or interaction with other factors to mediate a Hb switch; in K562 cells, transfected EKLF activates a cotransfected β-globin promoter but is unable to activate the endogenous, chromosomally located β-globin gene (9). Thus, important in this developmental story are gene regulatory factors yet to be discovered that are themselves developmentally regulated, e.g., expressed in embryonic erythroid cells but not in adult erythroid cells and vice versa (1).

Human K562 cells treated with hemin have characteristics of embryonic erythroid cells, including expression of ε- and γ-globins, but no adult β-globin, and expression of ferritin enriched in H-chains (10, 11). Erythrocytes of adults express much less ferritin (12) that is relatively more enriched in L-chains (13). It has been reported that human ε- or γ-globin promoter-reporter gene constructs are stimulated by cotransfected ferritin-expressing cDNA clones in HeLa cells (14). In contrast, expression of the adult β-globin gene is inversely correlated with that of ferritin-H.

In addition to the known role of ferritin as a cytoplasmic iron storage protein (12, 15, 16), recent reports show that ferritin-H is also a nuclear protein in K562 and other cells, including mammalian neurons (17–21). Even so, the role we postulate for ferritin as a gene regulator in Hb switching is unexpected. Ferritins exist primarily as large heteropolymers of 24 subunits in animal cells (12, 16). Both H and L subunits are required to efficiently store iron, H-ferritin having ferroxidase activity (to convert Fe^{2+} to Fe^{3+}, the storage form) and L-ferritin containing nucleation sites for Fe^{3+} that face the cavity of the polymer and allow aggregates of up to 4,500 Fe atoms to form (12, 15, 16). Single subunits or dimers of H-subfamily chains are postulated to be the form of ferritin that enters the nucleus. It is noteworthy that bacterial ferritins having structural homology with mammalian ferritins are DNA-binding proteins; dual functions of iron- and DNA-binding evolved early (22–24).

In light of these findings, we hypothesize that nuclear ferritin acts as a gene regulator with the ability to repress the human adult β-globin gene. We show that a ferritin-family protein in nuclear extracts of K562 cells can bind the 5′-β-globin promoter between −153 and −148 bp from the cap site and that a highly conserved hexanucleotide sequence, CAGTGC, is required for this binding. Ferritin-H binds this sequence better than ferritin-L and represses β-globin promoter function in cotransfection assays; and the −150 CAGTGC motif is required for the repressor activity of ferritin-H. In previous studies with MEL cells, the CAGTGC motif appears to be a focal point for both positive and negative regulation and is part of the binding site of a purported repressor of adult mouse β-globin (25, 26). A β-globin gene repressor could be useful in ameliorating sickle cell and other genetic diseases because a compensatory increase in γ-globin(fetal) expression can be expected when β-globin is decreased (27).

Materials and Methods

Cell Lines. K562 human erythroleukemia cells, used for nuclear extracts (28), and CV-1 African green monkey kidney epithelial cells, used for transfections (29), were grown in RPMI medium 1640 and DMEM, respectively, with 10% FBS and antibiotics.

Proteins and Antibodies. Ferritins from human liver (rich in L chains) and heart (rich in H chains), human transferrin, apotrans-

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Abbreviations: CAT, chloramphenicol acetyltransferase; EKLF, erythroid Krüppel-like factor; wt, wild type; GFP, green fluorescent protein.

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ferrin, and polyclonal antiserum to human spleen ferritin were from Sigma.

Restriction Fragments and Oligonucleotides. The 5′ region of the human β-globin gene (−610 to +20 bp) was cut from its cloned site in pSV2CAT (28) with *Hin*dIII and *Rsa*I, giving three fragments, −638/−223 (416 bp), −222/−128 (95 bp, distal promoter), and −127/+20 (147 bp, proximal promoter), that were end-labeled with ^{32}P as described (30). Synthetic oligonucleotides of both strands of the −232/−188 and −164/−128 regions were purified, annealed (28), and used as unlabeled competitors or labeled probes in gel mobility-shift assays.

Preparation of Nuclear Extracts. Nuclear extracts were made from cultures of K562 cells (1×10^6 cells per ml) by the procedure of Dignam *et al.* (31); protein content ranged from 3 to 6 mg/ml. Extracts with ferritin-family protein(s) 80–90% pure were prepared by treating the crude extracts with proteinase K and/or heat at 75°C (32).

Gel Mobility-Shift Assays. Gel-shift assays (electromotive shift assays) were used to determine binding of extract proteins to the *Rsa*I (95-bp) fragment and synthetic oligonucleotides (28, 33). Typically, a binding reaction at room temperature contained 0.5–2 ng of DNA, 1.0–5.0 μg of crude nuclear extract protein, 1.0–5.0 μg of poly(dI)·poly(dC), 100 mM KCl, and binding buffer (28). Unlabeled competitor oligonucleotides ranged from 15- to 1000-fold molar excess, and were included in the reaction mixture with the probe before adding protein. Gels used for retardation assays were 4 or 6% acrylamide (29:1, acrylamide:bisacrylamide) with glycerol in the gel; running buffer was low ionic strength TAE (28).

Sequence Alignments and Homology Searches. Mammalian β-globin promoter sequences (−200/+1) were obtained from GenBank and manipulated by using the PILEUP and LINEUP programs of the University of Wisconsin Genetics Computer Group (Madison, WI) package.

Clones, Transfections, and Gene Expression Assays. The upstream region (−610/+20) of the human β-globin gene, previously cloned in pSV2CAT (28), was subcloned through pGEM and pSELECT (now called pALTER) and recloned into pCAT-basic (all vectors from Promega). Mutants of the −153/−148 promoter site were generated by transcription from mutant oligonucleotides corresponding to the −164/−128 region by using the pSELECT system. Transfections of CV-1 cells with DMRIE-C reagent (GIBCO/BRL) in OptiMEM serum-free medium were optimized by using green fluorescent protein (GFP) plasmid pEGFP-C1 (CLONTECH). Expression of GFP was followed by fluorescence microscopy and quantitative fluorescence of cell lysates. The reporter gene chloramphenicol acetyltransferase (CAT) was measured in transfected cell lysates by an ELISA (Promega) standardized with purified CAT. The EKLF effector clone is in pSG-5 (Stratagene; ref. 29) and the ferritin-H expression clone is in the eukaryotic expression vector pcEXV-1 (14). Total cellular protein was measured with the bicinchoninic acid assay (Pierce) using BSA as a standard.

Results

A Ferritin-Family Protein in K562 Cell Nuclear Extracts Binds the −164/−128 Region of the β-Globin Promoter. By using polyclonal antisera to human spleen ferritin, an antibody supershift assay was performed with crude K562 nuclear extract and ^{32}P-labeled fragments of the proximal (−127/+20) and distal (−222/−128) human β-globin promoter, as well as two overlapping synthetic oligonucleotides diagrammed in Fig. 1*a*. Initial experiments showed that only the distal promoter (−222/−128) was bound by nuclear extract protein that reacted with anti-ferritin antiserum, consistent with

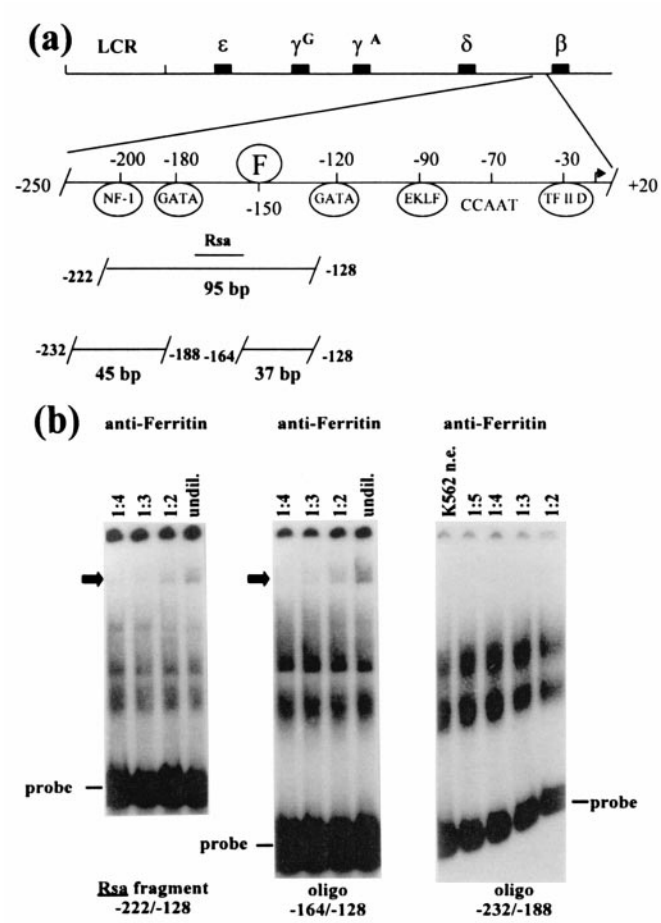


Fig. 1. Nuclear anti-ferritin-reactive protein binds the β-globin promoter. (*a*) Diagram of the human β-globin locus, a 5′ region of the β-globin gene (−250 to +20), and specific segments used in *b*, i.e., the distal promoter (−222/−128) and two double-stranded oligonucleotides, −164/−128 and −232/−188. (*b*) Binding of a ferritin-family protein from K562 cell nuclear extracts to the −222/−128 β-globin region, and localization of the binding to the −164/−128 segment by using an antibody supershift assay described under *Materials and Methods*.

reports of the importance of these sequences for both activation (25) and repression (26). As shown in Fig. 1*b*, the left series of lanes, a single supershift band was obtained (arrow) with the 95-bp distal promoter that increased in intensity with increasing antiserum. To further localize the binding of the anti-ferritin reactive protein, the supershift was also performed with ^{32}P-labeled double-stranded oligonucleotides of the −232/−188 and −164/−128 sequences. The more 3′ oligonucleotide gave a supershift band, whereas the more 5′ oligonucleotide did not (Fig. 1*b*), indicating that the protein recognized by the antiserum binds to a 37-bp sequence between −164 and −128. The lack of a supershift with the −232/−188 oligonucleotide also serves as a control for the specificity of the antibody; other controls are described below.

The Distal Promoter-Binding Protein Has Other Properties of Ferritin. Ferritin, unlike most proteins, is resistant to proteinase K digestion and temperature to 75°C and can be obtained 90% pure from extracts of embryonic erythroid cells by using these treatments (32). When this procedure was applied to K562 nuclear extracts, several proteins that yield bandshifts were depleted, but the remaining protein gave a single shift band with a −222/−128 restriction fragment (Fig. 2, lane 3). Addition of anti-ferritin antiserum after preincubation of the DNA and binding protein produced a sub-

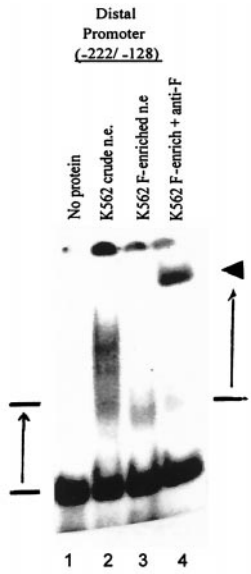
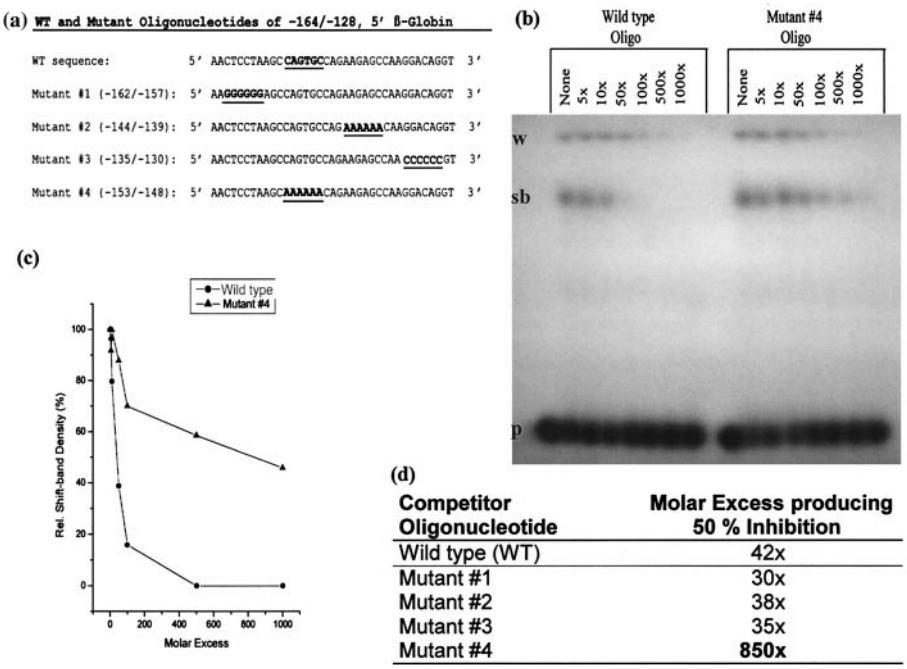


Fig. 2. The β-globin promoter-binding protein has three properties of ferritin (proteinase K resistance, heat stability, and reactivity with anti-ferritin antiserum). A method to obtain ferritin 90% pure from embryonic red cells by using proteinase K digestion followed by treatment at 75°C (32) was applied to K562 nuclear extracts. The clear supernatant fluid obtained by centrifugation gave a single shift band with the $-222/-128$ promoter (lane 3) and a ''supershift'' with anti-ferritin antiserum (lane 4). The arrows at the left and right sides of the figure show the relative distances the DNA is shifted in the primary and supershift reactions, respectively.

stantial supershift (Fig. 2, lane 4). Arrows at the left and right sides of Fig. 2 denote the degree that DNA mobility is retarded in the primary shift and the supershift, respectively. These results show that K562 nuclear extracts contain a protein that has three properties of ferritin—resistance to proteinase K digestion, stability to heat at 75°C, and reactivity with anti-ferritin antisera—and that this protein binds to the $-222/-128$ promoter region of β-globin DNA. Further, proteinase K and/or heat treatment can quickly partially purify the protein from crude nuclear extracts.

The primary shift band obtained after proteinase K digestion is lower on the gel than shift bands obtained with untreated extract. Nuclear ferritin is partially digested by proteinase K to a degree dependent on both time and amount of enzyme, consistent with a previous report (32). We find that the protein that remains after a 10- to 15-min digestion (Fig. 2, lane 3) is a proteinase K-resistant core that still binds DNA. Controls show that the supershift is specific for ferritin epitopes and for this DNA–protein complex (e.g., complexes with other DNAs, such as β-IVS2, are not supershifted; no supershift occurs with nonimmune rabbit serum, depletion of extracts with anti-ferritin antiserum and protein-A-agarose beads removes the material that causes the primary shift band, and purified ferritin blocks the ability of the antiserum to cause a supershift).

Competition Gel Shift Assays Define a CAGTGC Motif at $-153/-148$ Required for Nuclear Ferritin Binding. To further map the binding site of the nuclear ferritin, we mutated the 37-bp oligonucleotide in different places, replacing six nucleotides at a time with all As, Cs, or Gs, including complementary nucleotides in the opposing strand (Fig. 3*a*). Competition gel shifts were done with the partially purified protein from heated K562 nuclear extract, such that unlabeled mutant oligonucleotides and the unlabeled wild type (wt) competed against the ^{32}P-labeled wt sequence for binding. All mutants competed for binding as well as wt except those mutated in the $-153/-148$ region (i.e., in the CAGTGC motif), which requires 20 times as much oligonucleotide to compete away the labeled shift band (Fig. 3*b*). The amount of label in all shift bands was quantified by densitometry, and the results corresponding to Fig. 3*b* are plotted in Fig. 3*c*. The results for all mutants, expressed as molar excess required to produce 50% inhibition of the radioactive shift band, are summarized in Fig. 3*d*. The binding to single-stranded oligonucleotides of any of the sequences was found



Competitor Oligonucleotide	Molar Excess producing 50 % Inhibition
Wild type (WT)	42x
Mutant #1	30x
Mutant #2	38x
Mutant #3	35x
Mutant #4	**850x**

Fig. 3. Definition of the binding site of nuclear ferritin, by using oligonucleotide competition assays. (*a*) Sequences of wt and mutant oligonucleotides corresponding to the 5′ binding region mapped in Fig. 1 are shown. Mutated nucleotides and the original CAGTGC sequence are underlined. These oligos, used in the competitive gel shifts in *b*, *c*, and *d*, were double-stranded; only the top strands are shown. (*b*) Competition gel-shift assays using the end-labeled wt sequence versus unlabeled wt or mutant no. 4 oligonucleotides, with partially purified ferritin-protein from K562 nuclei. Unlabeled oligonucleotides in the fold excesses shown were present with the labeled wt sequence at the time binding was initiated. Whereas the wt sequence competes significantly with itself at 50-fold excess, the oligonucleotide mutated in the CAGTGC sequence requires 1,000-fold excess to give the same level of competition. Labels: p, probe; sb, shift band; w, wells. (*c*) Relative optical densities of the shift bands plotted versus molar excess of competitors, for the gel in *b*. (*d*) Molar excesses of wt and mutant oligonucleotides required to produce 50% inhibition of binding of the labeled probe. Each mutant oligonucleotide required about the same molar excess as the wt sequence to produce 50% inhibition except mutant no. 4 (mutated in the $-153/-148$ CAGTGC sequence), which required a 20-fold greater concentration to compete to the same extent, indicating that CAGTGC is crucial for the DNA–protein interaction.

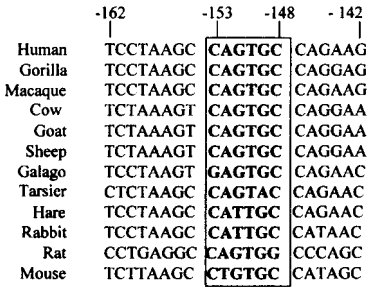


Fig. 4. Multiple sequence alignment of mammalian β-globin promoters, showing the high degree of conservation of the CAGTGC motif. Promoter sequences (corresponding to $-162/+1$ of the human β-globin gene) were aligned for 12 mammalian species. GenBank accession nos. for the sequences are (from top down) V01317, X61109, X05665, X00376, M15387, X14727, M61740, J04429, Y00347, M11818, X15009, and X14061. Alignments were generated by using the programs PILEUP and LINEUP.

to be nonspecific. We conclude that the CAGTGC motif is required for binding of K562 cell nuclear ferritin to the β-globin promoter.

Sequence Alignments of 12 Mammalian Adult β-Globin Promoters Show That the -150 CAGTGC Motif Is Highly Conserved. In a phylogenetic comparison of 12 mammalian adult β-globin promoters from -162-bp to the $+1$-cap site, the -150 region CAGTGC sequence (Fig. 4) is among the most conserved of the cis-acting elements, second only to the TATA and CCAAT boxes in its high degree of conservation, and as highly conserved as the proximal and distal CACCC motifs.

Purified Ferritin Binds to β-Globin Promoter DNA. The distal promoter of the human β-globin gene ($-222/-128$ bp) is bound by human liver or heart ferritin, as shown in gel-shift assays (Fig. 5, lanes 3 and 4), whereas the proximal promoter is not (Fig. 5, lanes 7–9). H-chain-rich heart ferritin (lane 4) showed a higher degree of binding than L-chain-rich liver ferritin (lane 3). Controls—another iron-binding protein, transferrin, and its iron-free form, apotransferrin—showed no binding to the distal fragment bound by ferritin (Fig. 5, lanes 5 and 6).

The dark bands in the wells of lanes 2–4 and 7–9 may represent probe bound by the 24-mer ferritin shells that cannot enter the gel because of their size, whereas the specific shift bands within the gel likely result from ferritin subunits that dissociate from the shells in the DNA-binding buffer. As subunits bind DNA, their tendency to dissociate from the otherwise stable shells may increase, especially at the 37°C temperature used for this set of binding reactions. Thus, more binding by ferritin-H results in both a darker well and a darker shift band for heart ferritin than for liver ferritin. It is not possible to do this experiment with native ferritin subunits because the amount of detergent required to dissociate ferritin oligomers and keep them apart is incompatible with DNA binding.

Ferritin-H Represses the β-Globin Promoter in Cotransfection Assays, and Mutation of the -150 CAGTGC Motif Abolishes Repression by Ferritin-H. A transient expression assay was set up with CV-1 cells (29), in which β-globin promoter-driven expression of a reporter gene is low unless the cells are cotransfected with an expression clone of EKLF, a specific activator of β-globin transcription (9). Results with a wt β-CAT (W) reporter plasmid are shown in Fig. 6, in the left-hand set of bars. EKLF-stimulated β-CAT (W) expression (hatched bar) was repressed in excess of 60% by cotransfection with human F_H expression clones (solid bar).

Controls included a positive CAT control plasmid that expresses constitutively, and a negative CAT basic plasmid that contains no promoter. The experiment in Fig. 6 was repeated five times with

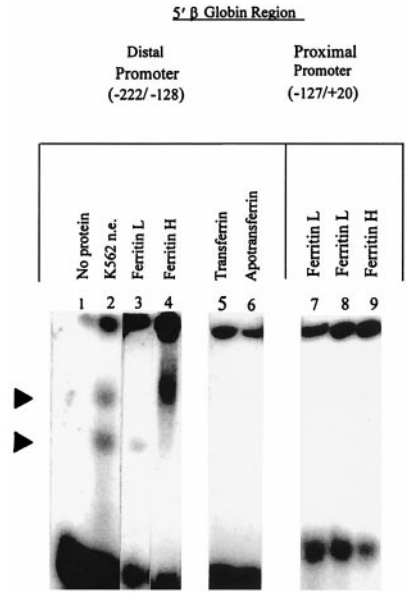


Fig. 5. Binding of ferritin chains from human heart and liver to the distal promoter of the human β-globin gene. The *Rsa* fragment of the β-globin promoter ($-222/-128$) was end-labeled with ^{32}P and 1 ng of DNA was used as a probe in electromotive shift assays as described (28), with 1 μl of K562 nuclear extract (lane 2) or 2–4 ng of purified proteins, as follows: 2 ng of human liver ferritin (lane 3), 4 ng of human heart ferritin (lane 4), 2 ng each of human transferrin (lane 5) and apotransferrin (lane 6). Shift bands are denoted by arrowheads at the left. Lane 1 contained only DNA. A second fragment of the β-globin promoter ($-127/+20$) gave no shift bands with either 2 ng (lane 7) or 4 ng (lane 8) of liver ferritin or 4 ng of heart ferritin (lane 9). Only ferritin enriched in H-chains gave a strong shift (lane 4). The DNA-binding reactions for this figure were carried out at 37°C.

β-CAT and three times with a β-luciferase construct (9) with very similar results. The repression is also evident (although reporter activity is much lower) when EKLF is omitted (data not shown). Other controls included cotransfection of "empty" carrier plasmids for all clones (no effect on reporter gene expression). Cotransfection of ferritin-L sometimes resulted in repression; but the effect was both less dramatic and inconsistent.

We also constructed both wt and mutant β-globin promoters at the -150 CAGTGC motif, linked each to a CAT reporter, and tested each construct for its ability to support repression by ferritin-H in this cotransfection assay. As also shown in Fig. 6, in the right-hand set of bars, we find that the mutant β-CAT (M) promoter is stimulated by cotransfected EKLF (hatched bar) as well as the native promoter, whereas ferritin-H repression is lost—i.e., the β-CAT (M)/EKLF/F_H (solid bar) is as high or higher than the β-CAT (M)/EKLF (hatched bar). The -150 mutated β-CAT (M) plasmid (Fig. 6) is an ideal control because everything is identical except a short sequence of nucleotides in a specific region, i.e., the CAGTGC motif; the fact that EKLF stimulation is retained with this plasmid shows that there is no nonspecific repression due to overexpression of ferritin-H. Thus, mutation of the -150 CAGTGC motif not only markedly reduces *in vitro* binding of nuclear ferritin (Fig. 3) but also abrogates the ability of expressed ferritin-H to repress this promoter in our cell transfection assay, providing a strong link between DNA binding and function.

Discussion

Our results show that a ferritin-family protein in K562 cell nuclear extracts binds the distal promoter of the human β-globin gene (Fig. 2) between -128 and -164 bp (Fig. 1), that the binding specifically requires a CAGTGC motif at $-153/-148$ (Fig. 3) that is highly conserved (Fig. 4), and that native human

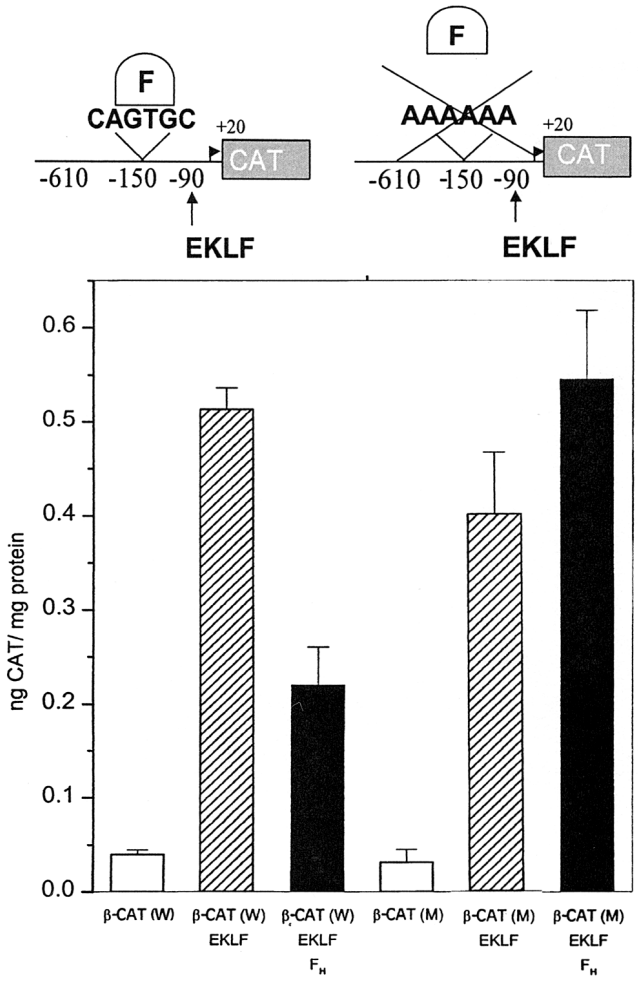


Fig. 6. Cotransfection experiments demonstrating ferritin-H repression of the β-globin promoter and loss of ability to repress when the ferritin binding site (CAGTGC) is mutated. Transfections of CV-1 cells were performed with a constant amount (6 μg) of total plasmid DNAs mixed with 8 μl of DMRIE-C added to 2×10^6 CV-1 cells, such that each transfection had 2 μg of β-CAT plasmid (W = wt, or M = mutant), ± 1 μg of EKLF, ± 3 μg of F_H (ferritin-H expression plasmid), with the difference made up to 6 μg with pEGFP. Reporter gene activity, expressed as ng of CAT per mg of cellular protein (measured by ELISA), is shown for the following combinations with either native (W) or mutant (M) β-CAT plasmids: the nonstimulated human β-globin promoter (open bars); the β-globin promoter stimulated by a cotransfected EKLF effector plasmid (hatched bars); and EKLF-stimulated β-globin promoter cotransfected with a ferritin-H expression plasmid (solid bars). (n = 3 transfections per data set; bars = SEM). Construction of reporter plasmids (diagrammed above the histogram) is described in *Materials and Methods*.

ferritin-H also binds this DNA sequence (Fig. 5). This binding was linked to function in cotransfection experiments with CV-1 cells: ferritin-H represses EKLF-stimulated β-globin promoter-reporter expression, and mutation of the -150 CAGTGC ferritin-H binding site abrogates this repression without affecting EKLF stimulation (Fig. 6). Thus, both "gain of function" and "loss of function" were demonstrated with our cotransfections.

The CAGTGC motif required for nuclear ferritin binding and repression of β-globin occurs in the human β-like globin gene cluster at 3 times the frequency expected by chance, including the 5′ and 3′ flanking regions of the embryonic (ε)- and fetal (γ)-globin genes—e.g., ≈ -1070 of both γs. However, the locations and sequence context in which CAGTGC and similar sequences (i.e., CAGTGN) are found differ from the adult β-globin gene where the motif functions as a repressor. For example, the sequence CAGTGA is found 5′ of the $^G\gamma$ gene at -1043 just upstream of an E-box‖, as well as at -789 and -69, where it abuts GATA-1 sites in the opposite strand. It is not known whether nuclear ferritin binds these other sites, but published work (14) suggests that such binding would lead to ε- and/or γ-globin gene activation, in line with nuclear ferritin's developmental role as a β-globin repressor as postulated here. The human β-gene -150 CAGTGC oligonucleotide competes in gel shifts with the homologous mouse β-major site reported to bind an uncharacterized repressor (26); CAGTGC motifs are also found in promoters of chicken and frog adult β-globin genes.

The CAGTGC motif is found in antioxidant-responsive elements (AREs) that mediate induction of a battery of genes in a chemoprotective response system (34), in DNA sequences preferentially sensitive to iron-mediated cleavage (35), and as an RNA copy in the consensus sequence at the apex of the stem-loops of iron-responsive elements (IREs) that are focal points for translational control in iron metabolism (36). It is intriguing that a nucleotide sequence important in iron metabolism is a focal point for repression of a gene (β-globin) that is a primary utilizer of iron.

The nuclear ferritin-family protein described here is distinct from other known trans-acting proteins in its physical properties as well as in its proposed function as a direct repressor of the β-globin promoter. The mediator of β-globin promoter binding and repression behaves as a ferritin-H subunit or dimer in gel-shift assays. We do not yet know whether the K562 cell nuclear-extract protein is a new member of the H-ferritin gene subfamily or is identical to the isolated and characterized ferritin-H (12, 16), nor do we know how the gene regulatory function of nuclear ferritin is tied to iron metabolism. The H-ferritin subfamily is represented by a larger number of genes than the L-ferritin subfamily (15, 16) and includes a cluster of genes and pseudogenes on the X chromosome (37). Until the native protein is isolated from K562 cell nuclear extracts and sequenced directly, its precise identity among ferritin subfamily members remains open.

Another interesting possibility is that the *in vivo* binding to the β-globin -150 CAGTGC motif includes a ferritin-associated protein (38) that would be protected from proteinase K and heat and be included in the reaction with anti-ferritin antisera because of its strong association with ferritin. Beaumont and coworkers (20) have reported that a protein from crude K562 cell nuclear extracts with a molecular weight greater than that of a ferritin subunit associates with this region of the β-globin promoter in UV-crosslinking experiments. However, the SDS gel depicted in figure 6 of their paper also shows a low molecular-weight staining region that could contain a ferritin peptide or proteolyzed fragment of ferritin-H. It is clear from our data that nuclear ferritin can bind the -150 site *in vitro*, possibly by way of a basic region in its C α-helix; preliminary data with BIACORE surface plasmon resonance measurements (V.B. and R.H.B., unpublished data) indicate that the partially purified K562 cell nuclear ferritin binds the 37-bp oligonucleotide containing the -150 region CAGTGC motif with kinetics that model as single entity binding and a K_d value of $\approx 2 \times 10^{-10}$ M. However, our data do not exclude that another protein(s) could be part of the *in vivo* complex.

In eukaryotes multiprotein complexes are required for regulating gene transcription, and changes in conformation or composition of such complexes can mediate repression versus activation (39). The DNA-binding site of the ferritin-family protein we have discovered is homologous with a region required for activation and repression of β-globin in MEL cells (25, 26). Subsequent interaction of this binding site with upstream negative regulatory regions (28) could

‖A conserved element termed an E-box was first observed by Hardison [ref. 45 (cited in ref. 1)] and may be important in the enhancing action of HS2 of the β-globin LCR by way of the action of basic helix–loop–helix proteins such as TAL1, USF, and/or HS2NFE5. The role of the E-box in $^G\gamma$ gene expression is not known.

create a tightly bound complex that prevents binding of other positive factors such as GATA-1, and sterically hinders the formation of an active transcription complex on the proximal promoter by DNA looping. Local promoter-based regulation such as this is likely to be a key to developmental Hb switching; these promoter-based events may or may not necessitate direct interaction with the β-cluster locus control region, a question that is currently being debated (40, 41). The mechanism by which the β-globin gene repression reported here actually occurs will require further investigation using technology capable of identifying all proteins in a multisubunit complex, such as matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) mass spectrometry and peptide mass fingerprinting (42). It will also be of interest to determine whether ferritin-H will repress human β-globin and/or activate γ-globin sufficiently in a transgenic mouse model (43) to alleviate the sickle cell phenotype.

Although the existence of nuclear ferritin and the role we have proposed for it as a gene regulator have not been described previously, the discovery more than a decade ago that iron responsive element-binding protein-1 is a conformer of *cis*-aconitase, a metabolic enzyme, made it clear that proteins may have dual functions within the cell (36). Our data for nuclear ferritin match at least 6 of 12 criteria often applied to demonstrate the functional importance of a protein–DNA interaction (44). Furthermore, ferritin-H itself is expressed in a developmentally specific (and appropriate) manner in erythroid cells consistent with globin gene activities, i.e., it is expressed in embryonic but not adult, erythroid cells (13, 16). Thus, nuclear ferritin may be one of the long-sought switching factors and be useful in treating sickle cell and other genetic diseases.

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