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A CTCF-Dependent Silencer Located in the Differentially Methylated Area May Regulate Expression of a Housekeeping Gene Overlapping a Tissue-Specific Gene Domain

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The tissue-specific chicken α-globin gene domain represents one of the paradigms, in terms of its constitutively open chromatin conformation and the location of several regulatory elements within the neighboring housekeeping gene. Here, we show that an 0.2-kb DNA fragment located ~4 kb upstream to the chicken α-globin gene cluster contains a binding site for the multifunctional protein factor CTCF and possesses silencer activity which depends on CTCF binding, as demonstrated by site-directed mutagenesis of the CTCF recognition sequence. CTCF was found to be associated with this recognition site in erythroid cells but not in lymphoid cells where the site is methylated. A functional promoter directing the transcription of the apparently housekeeping *ggPRX* gene was found 120 bp from the CTCF-dependent silencer. The data are discussed in terms of the hypothesis that the CTCF-dependent silencer stabilizes the level of *ggPRX* gene transcription in erythroid cells where the promoter of this gene may be influenced by positive *cis*-regulatory signals activating α-globin gene transcription.

The cluster of developmentally regulated chicken α-globin genes contains an embryonic (π) and two adult (α^A and α^D) globin genes (Fig. 1A). Although expressed in erythroid cells only, this cluster (including an extended upstream area) resides in a DNase-sensitive chromatin domain in cells of different lineages (7). This might be a consequence of the location of the α-globin gene cluster within a gene-rich genomic area. Particularly, a housekeeping gene just upstream to the α-globin gene cluster and transcribed in the opposite direction in humans (46, 47), chickens (13, 42, 46), and mice (21, 22) has been described, and it is of interest that the major regulatory element of the α-globin gene cluster resides within one of the introns of this housekeeping gene (13, 22, 47). Hence, the domain of α-globin genes constitutes a classical example of a tissue-specific domain with vague boundaries that overlaps another gene domain (for a review, see reference 32). The regulatory systems of genomic domains of this type are quite complex and, as yet, poorly understood (9, 32). In a recent study, we described a group of CTCF-dependent enhancer-blocking elements located between the chicken α-globin cluster and its major regulatory element (44).

The purpose of the present study was to find out if any regulatory elements distinct from promoters ensure functional isolation of the chicken *ggPRX* gene from the α-globin gene cluster. In particular, we were interested in determining if the promoter-enhancer of the *ggPRX* gene contains regulatory elements stabilizing its expression in the course of erythroid differentiation, i.e., in a situation when a strong upstream enhancer can reach the *ggPRX* gene promoter and an erythroid-specific domain of hyperacetylated histones is established including the promoter and a significant part of the *ggPRX* gene (1). Using computer-assisted analysis, we found a homolog of the human "*-14*" gene promoter and a potential CTCF-binding site located in close vicinity within the chicken α-globin gene domain's upstream CpG-rich region (2.5 to 5 kb upstream of the embryonic α-type globin gene π). This CpG-rich region was previously found to contain a complex of functional elements including a replication origin (35, 45), a constitutive non-tissue-specific enhancer (34), and a differentially methylated area (33). In this study, we were interested in CTCF as one of the players possibly taking part in regulation of *ggPRX* gene expression. CTCF, a 11-zinc-finger CCCTC-binding factor, was first described as a transcription factor regulating c-*myc* gene activity (12, 23, 26) and was later demonstrated to be a versatile transcriptional regulator that may be involved in function of enhancers, silencers, and insulators (2, 10, 19, 28, 30). Taking into consideration an essential role of CTCF in enhancer-blocking activity in many previously characterized enhancer-blocking elements of vertebrates (2, 3, 16, 44) and *Drosophila melanogaster* (28), we tried to find out (i) whether the putative CTCF-binding site identified by computer-assisted analysis really binds CTCF and (ii) whether the DNA fragment bearing this site possesses enhancer-blocking activity. We demonstrated that CTCF does interact with this binding site in vitro and in vivo (in erythroid cells) and that a 200-bp DNA fragment bearing the CTCF-binding site possesses a silencer activity dependent on CTCF binding. Furthermore, it was

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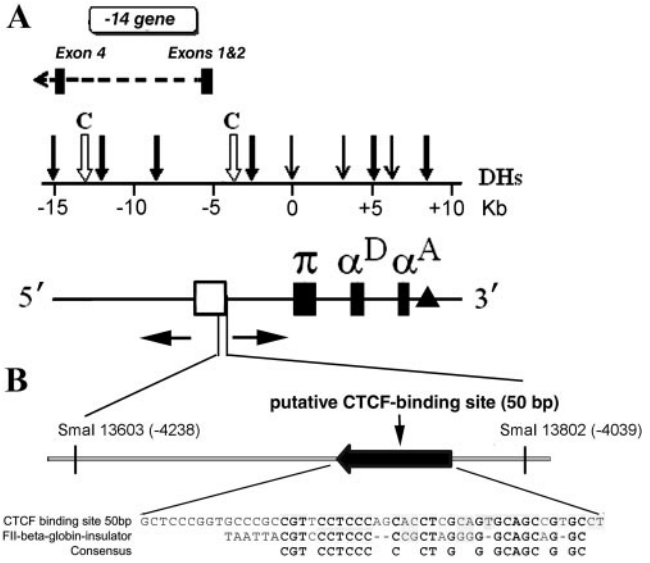

FIG. 1. (A) Schematic representation of the chicken α-globin domain with regulatory and structural elements. Globin genes and regions of homology with exons of the human gene "*-14*" are shown by filled rectangles. The open rectangle shows the position of the CpG island (33), which includes a weak enhancer (34) and an origin of bidirectional DNA replication (35, 45). Directions of replication are shown below by horizontal arrows. The filled triangle shows the position of a strong erythroid cell-specific enhancer (25) and silencer (38). DNase I-hypersensitive sites (DHs) are shown by arrows (15, 20, 31, 43); closed arrows represent erythroid cell-specific DHs; thin arrows show DHs in promoter regions; open arrows show constitutive DHs. (B) The putative CTCF-binding site (50 bp) located within the 200-bp SmaI fragment. The arrow indicates the direction of *ggPRX* gene transcription. The regions critical for CTCF binding deduced from alignment of the putative CTCF-binding site with the FII sequence (CTCF) recognition site from the chicken β-globin domain 5′ HS4 insulator (2) are shown in boldface type. The numbers defining the two SmaI sites correspond to GenBank sequence AF098919, and the numbers in brackets show their positions relative to the start of π gene transcription.

demonstrated that CTCF binding is prevented by methylation of this recognition site in lymphoid cells.

MATERIALS AND METHODS

Plasmids and constructs. The recombinant clone containing the 1.7-kb α62 delta DNA fragment of the chicken α-globin domain harboring the area of interest has been described previously (18). A 200-bp SmaI subfragment containing the putative CTCF-binding site was cloned in the SalI or HindIII site of pAcatE (37, 41) in both orientations. A 200-bp fragment with mutated CTCF-binding site was prepared in the following way. An oligonucleotide duplex containing the mutated sequence (see Fig. 6A) was ligated via the PstI-site to a PCR-amplified fragment representing the rest of the native 200-bp fragment and then cloned in the SalI site of pAcatE. The following oligonucleotides (purified by polyacrylamide gel electrophoresis) were used: for annealing a duplex with a mutated CTCF recognition site (only one of the complementary chains is shown), 5′-GGCTGCAGCTTGCTCCCGGTGCCCGCCGTTCCTAGCACC CAGTGCAGCCGTGCCTGAAGTGCAGCCCAGCACGTCGACTA-3′; for PCR amplification, dir (5′-TTGTCGACGGGGGCAGGTCGCGCTCAG AGGCC-3′) and rev (5′-AGCTGCAGCCGTGGGGCACCCCGAGGGCGT-3′) were used.

The promoters of the chicken *ggPRX* gene and the human *-14* gene were PCR amplified using the following primers: ggPRX prom-dir (contains a XhoI site) (5′-ACTACTCGAGGCAACGACGGACGCGGCTCC-3′), ggPRX prom-rev (contains a MluI site) (5′-ACTAACGCGTGAGCGCTGTGCCCGCAGCTC-3′), -14 prom-dir (contains a XhoI site) (5′-ACTACTCGAGACCGTCCGGAG GAGGTGCTA-3′), and -14 prom-rev (contains a MluI site) (5′-ACTAACGCG TATCCGCACCAGCAAGAGGGG-3′). The PCR-amplified 110-bp fragments were cloned in pCAT3 basic vector (Promega) via XhoI and MluI sites. Manipulations of recombinant DNA, agarose gel electrophoresis, and PCR amplifications were carried out as previously described (27).

Cell cultures. The avian erythroblastosis virus-transformed chicken erythroblast cell line HD3 (clone A6 of line LSCC) (4, 5) was grown in suspension in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 8% fetal bovine serum and 2% chicken serum. Chicken preerythroblast cell line 6C2 (avian erythroblastosis virus-transformed bone marrow cells arrested at the CFU erythrocyte stage) (4, 5, 44) was grown in minimal essential medium (Invitrogen) supplemented with 10% fetal calf serum, 2% chicken serum, 1 mM HEPES (pH 7.2), and 50 μM 2-mercaptoethanol. The DT40 lymphoid cell line was grown in DMEM supplemented with 50 μM 2-mercaptoethanol, 10% fetal calf serum, 5% chicken serum, and 10% tryptose phosphate broth. COS-1 cells (African green monkey kidney cells transformed by simian virus 40) and HeLa cells were grown in DMEM supplemented with 10% fetal bovine serum.

Transient transfection experiments. HD3 cells (2×10^6 to 3×10^6) and 5 μg of XmnI-linearized plasmid were used per transfection. In experiments with circular constructs, 2×10^6 to 3×10^6 HD3 or DT40 cells were transfected with 3 μg of plasmid. HeLa cells (10^6) were transfected with 1 μg of plasmid. Transfection efficiency was considered as previously described (44). Lipofectin (Invitrogen) was used for transfection according to the manufacturer's manual. Chloramphenicol acetyltransferase (CAT) activities were measured by liquid scintillation counting as described in the manufacturer's manual (Promega).

Chicken CTCF overexpression and preparation of nuclear extracts. Plasmid pSG5-CTCF (kindly provided by Elena Klenova) was transfected into COS-1 cells; after 72 h, cells were lysed by incubation for 2 min on ice in cell lysis buffer (10 mM NaCl, 20 mM HEPES [pH 7.6], 1.5 mM $MgCl_2$, 1 mM $ZnSO_4$, 20% glycerol, 0.1% Triton X-100, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) supplemented with Protease Inhibitor Cocktail (Sigma). After centrifugation, the pelleted nuclei were collected and shaken for 30 to 60 min on ice in nuclear extraction buffer (0.5 M NaCl, 20 mM HEPES [pH 7.6], 1.5 mM $MgCl_2$, 1 mM $ZnSO_4$, 20% glycerol, 0.1% Triton X-100, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) supplemented with Protease Inhibitor Cocktail (Sigma). After centrifugation ($7{,}000 \times g$; 10 min), nuclear extracts were stored at −70°C. The same protocol was used for preparation of nuclear extracts from HD3 cells.

EMSA. DNA fragments with 3′-recessed ends were end labeled with Klenow enzyme (Fermentas). Oligonucleotide duplexes were end labeled with T4 polynucleotide kinase (Fermentas). The electrophoretic mobility shift assay (EMSA) was performed as previously described (2, 14). In brief, binding assays were carried out on ice for 30 min in a buffer composed of 20 mM HEPES (pH 7.6), 40 mM NaCl, 5 mM $MgCl_2$, 0.5 mM $ZnSO_4$, 0.1 mM EDTA, and 4% Ficoll, with 100 to 200 fmol end-labeled DNA, 0.5 μg poly(dI-dC) used as a nonspecific competitor, and 5 to 6 μg nuclear protein extract in a final volume of 10 μl. When necessary, specific competitor DNA was added simultaneously with the labeled DNA at a 10- to 50-fold molar excess. The following oligonucleotides (purified by PAGE) were used in different mobility shift experiments (only one of the complementary chains is shown): FII (CTCF recognition site from chicken β-globin domain 5′ HS4 insulator) (2), 5′-CCCAGGGATGTAATTACGTCCCTCCCC CGCTAGGGGGCAGCAGGCGCGCCT-3′; C (putative CTCF recognition site studied in the present work), 5′-GCTCCCGGTGCCCGCCGTTCCTCCCAGCAC CTCGCAGTGCAGCCGTGCCT-3′; FM, 5′-GCTCC^{met-}CGGTGCC^{met-}CGC^{met-} CGTTCCTCCCAGCACCT^{met-}CGCAGTGCAGC^{met-}CGTGCCT-3′; M1, 5′-GCTCC^{met-}CGGTGCCCGCCGTTCCTCCCAGCACCTCGCAGTGCA GCCGTGCCT-3′; M2, 5′-GCTCCCGGTGCC^{met-}CGCCGTTCCTCCCAGCAC CTCGCAGTGCAGCCGTGCCT-3′; M3, 5′-GCTCCCGGTGCCCGC^{met-} CGTTCCTCCCAGCACCTCGCAGTGCAGCCGTGCCT-3′; M4, 5′-GCT CCCGGTGCCCGCCGTTCCTCCCAGCACCT^{met-}CGCAGTGCAGCCC GTGCCT-3′; and M5, 5′-GCTCCCGGTGCCCGCCGTTCCTCCCAGCAC CTCGCAGTGCAGC^{met-}CGTGCCT-3′.

Western blots. Nuclear protein extracts were quantified using the Bradford assay kit (Sigma). After separation by sodium dodecyl sulfate (SDS)-PAGE, proteins were transferred to polyvinylidene difluoride membranes (Amersham Biosciences), which were blocked with 5% nonfat milk overnight at 4°C and then incubated with anti-CTCF antibody (ab10571; Abcam) for 2 h at room temperature. After washing, the blots were incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G secondary antibody (Amersham Biosciences) and washed, and CTCF was detected with a chemiluminescence substrate (Amersham Biosciences) according to the manufacturer's instructions.

ChIP assay and PCRs. Chromatin immunoprecipitation (ChIP) was done as previously described (44). Briefly, 10^7 cells were incubated with 1% formalde-

hyde to cross-link the DNA and bound proteins, lysed by SDS, and sonicated to obtain chromatin fragments ranging in size from 100 bp to 500 bp. Samples from 10^6 cells were used for each immunoprecipitation with either a rabbit anti-chicken CTCF serum or a rabbit preimmune serum as a negative control (44). PCR was performed with the following primers: for the CG-rich region, SRF1 (5′-CGGGCAGGTCGCGCTCAGAG-3′) and SRR1 (5′-CGGGGCTGAGGT GTGAGGTG-3′); for β^A/ε enh, 0026 (5′-CTGGGTGGGGGCAGGT-3′) and 0027 (5′-CTGCTTTTGCTGCCCTGTG-3′). PCRs were carried out with HotStart *Taq* DNA Polymerase (QIAGEN) for 28 to 30 cycles in a standard mixture supplemented with 2 μCi of [α^{32}P]dCTP. The fragments were separated on a 6% polyacrylamide gel at 150 V in 0.5× Tris-borate-EDTA, and gels were dried and exposed to a storage phosphor screen (Amersham Biosciences).

RT-PCR analysis. Total nuclear RNA (0.5 μg) treated with DNase I (RNase free) (Roche) was reverse transcribed into cDNA using Moloney murine leukemia virus reverse transcription (RT)-polymerase (Fermentas). Two α-globin gene-directed primers located close to one another were used to start the RT reaction: primer 1 (5′-GCCCTCCCAAACCCCCTCTG-3′) and primer 2 (5′-C CTCTGGGTTCAGCAGCGCA-3′). The enzyme was omitted as a negative control. The cDNAs synthesized were treated with a mixture of RNase H and RNase A and amplified in 20 μl PCRs with an enzyme mixture containing thermostable *Taq* DNA polymerase (Fermentas) and proofreading *Pfu* DNA polymerase (Fermentas) in the presence of 1 M betaine (Sigma) and 5% dimethyl sulfoxide (Fermentas) and different sets of primers, listed below. PCR was performed for 28 cycles (each cycle consisting of 95°C for 45 s, a specified annealing temperature for 30 s, and 72°C for 30 s), preceded by denaturation at 95°C for 5 min. Ten-microliter aliquots of each reaction were electrophoresed on a 1.5% agarose gel prepared in 1× Tris-acetate-EDTA buffer. The following primers were used for amplification of different regions: test region 1 (246 bp), 5′-CACCAGGGCTGGCTGCACATG-3′ and 5′-GAGGAGGGGCGGCTC GAACC-3′; test region 2 (128 bp), 5′-CGCACTTACTGGCCTGGGC-3′ and 5′-CGAGAGCACCAGCCCCATC-3′; test region 3 (210 bp), 5′-ACGCTGATG GGGCTGGTGC-3′ and 5′-CCTCTGAGCGCGACCTGCC-3′; and test region 4 (161 bp), 5′-ACCCTCACCACAGCACAGC-3′ and 5′-TGAGTGGGGTGT GAGAGGTG-3′.

Sodium bisulfite treatment, PCR amplification, cloning, and sequence analysis. Genomic DNA from HD3 and DT40 cell lines was digested with SacI. For the time course study, 1 μg of digested DNA was denatured at 95°C for 5 min, chilled on ice, and incubated with 0.3 M NaOH in a 20-μl reaction volume at 37°C for 5 min. A freshly prepared solution of sodium bisulfite (S-8890; Sigma), adjusted to pH 5 with NaOH, and hydroquinone (H-9003; Sigma) were added at final concentrations of 1.7 M and 0.5 mM, respectively, in a final volume of 240 μl. DNA solutions were gently mixed, overlaid with mineral oil, and incubated at 55°C for 16 h. Unreacted bisulfite was removed by column chromatography (Wizard DNA Clean-Up System A7280 HR; Promega) as recommended by the manufacturer. Purified DNA samples were mixed with NaOH at a final concentration of 0.3 M, incubated at 37°C for 20 min, and neutralized on the DNA Clean-Up System. The flowthrough fraction (~30 μl) containing the converted DNA was stored at −20°C. Nested PCRs were performed in 50-μl reaction volumes using *Taq* DNA polymerase. For the first PCR amplification, 5 μl of bisulfite-treated DNA and 20-pmol/μl SRbisF1 (5′-GTGTTTTTAGT(C/T)GG TTGTTGT-3′) and SRbisR1 (5′-CTATACTATAATAAAAATATAAAAA-3′) primers were used. For the second PCR amplification, 5 μl of the first PCR amplification mixture and 20-pmol/μl SRbisF2 (5′-TTTT(C/G)GGGGTGTTT TA(C/G)GGTTGTTGT-3′) and SRbisR2 (5′-CTAAACTAAATAAAAAAC ACAAAAACTCAA-3′) were used. Amplification products were subjected to electrophoresis in 1.5% Tris-acetate-EDTA agarose gels and purified using the Qiaquick gel extraction kit (QIAGEN). The purified amplification products were ligated into plasmid pDrive cloning vector using the QIAGEN PCR cloning kit and transformed into competent *Escherichia coli* DH-5α by the $CaCl_2$ method. Colonies were screened on the basis of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining, and plasmid DNA was prepared by the alkaline lysis method. Plasmid clones harboring inserts of the appropriate size, as measured by restriction analysis, were amplified with the SP6 promoter primer and the ABI PRISM Dye Terminator cycle sequencing ready-reaction kit and sequenced with an ABI PRISM 377 DNA sequencer (Perkin-Elmer). The experiment was done at least twice, and 14 clones from each were analyzed.

RESULTS

Identification of a CTCF-binding site within the upstream CpG island of the chicken α-globin gene domain. To find potential CTCF-binding sites within the upstream CpG island of the chicken α-globin gene domain, we searched this area for the presence of regions sharing homology with the bona fide CTCF-binding site (known as FII) present within the upstream insulator of the chicken β-globin gene domain (2, 6). Computer-assisted analysis allowed us to identify three potential CTCF-binding motifs within the CpG island under study, including a putative 50-bp-long CTCF-binding site located in the downstream end of the ~200-bp SmaI fragment with coordinates 13603 to 13802 in sequence AF098919 (Fig. 1). The EMSA technique was used to determine if this fragment really bound CTCF. Electrophoresis and Western blotting confirmed that a nuclear extract from chicken HD3 cells contained endogenous CTCF; a 140-kDa protein band was revealed with CTCF-specific antibodies (Fig. 2B) that is consistent with previously published data on CTCF migration in SDS-PAGE (24). Incubation of the 200-bp DNA fragment with this nuclear extract in the presence of a vast excess of nonspecific competitor DNA resulted in the appearance of a retarded complex (Fig. 2A), which was effectively competed (at 10× molar excess) by a DNA fragment containing a bona fide CTCF-binding site from the chicken β-globin gene domain upstream FII insulator (Fig. 2A). In the

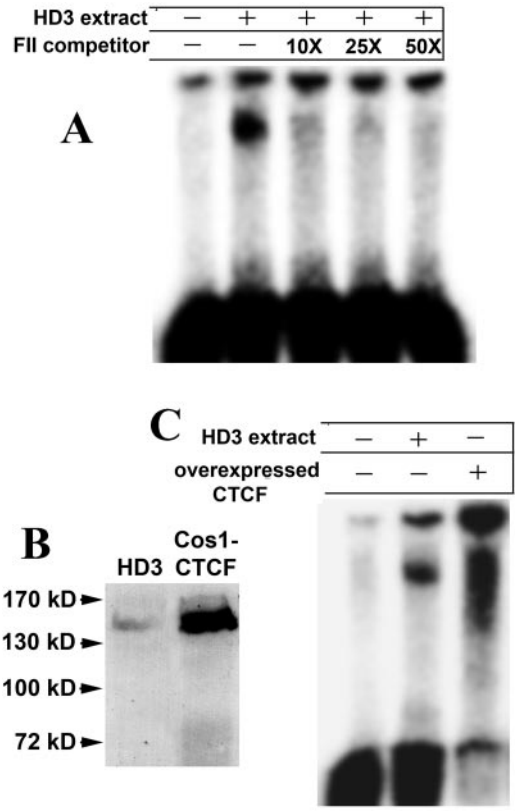

FIG. 2. CTCF interacts in vitro with the downstream part of the CpG island from the 5′ extended area of the chicken α-globin gene domain. (A) Electrophoretic mobility shift assay using nuclear extracts from HD3 cells. Note that the retarded complex was competed by a 10-fold molar excess of unlabeled FII DNA. (B) Western blot with CTCF-specific antibodies showing endogenous chicken CTCF in nuclear extracts of HD3 cells and overexpressed chicken CTCF in COS-1 cells transfected with a pSG5-CTCF vector. (C) Gel shift experiment with nuclear extract from HD3 cells or from COS-1 cells overexpressing chicken CTCF.

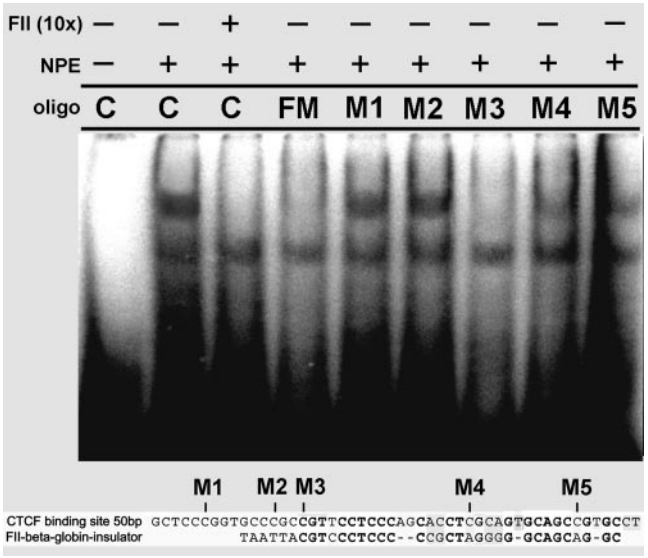

FIG. 3. Methylation interferes with CTCF binding to the recognition sequence under study. Gel retardation experiments with a radiolabeled 50-bp fragment containing the putative CTCF-binding site either nonmethylated (C), fully methylated at all five CpGs (FM), or methylated at individual CpGs (M1 to M5). Nuclear protein extract (NPE) from HD3 cells was added to all reaction mixtures, except that shown in lane 1. In lane 3, a 10-fold molar excess of specific inhibitor (FII unlabeled DNA) was present in the reaction mixture along with poly(dI-dC) (note that the upper band was specifically competed). The sequence of the putative CTCF recognition site is presented below with the positions of CpG dinucleotides (M1 to M5) that were either totally or individually methylated.

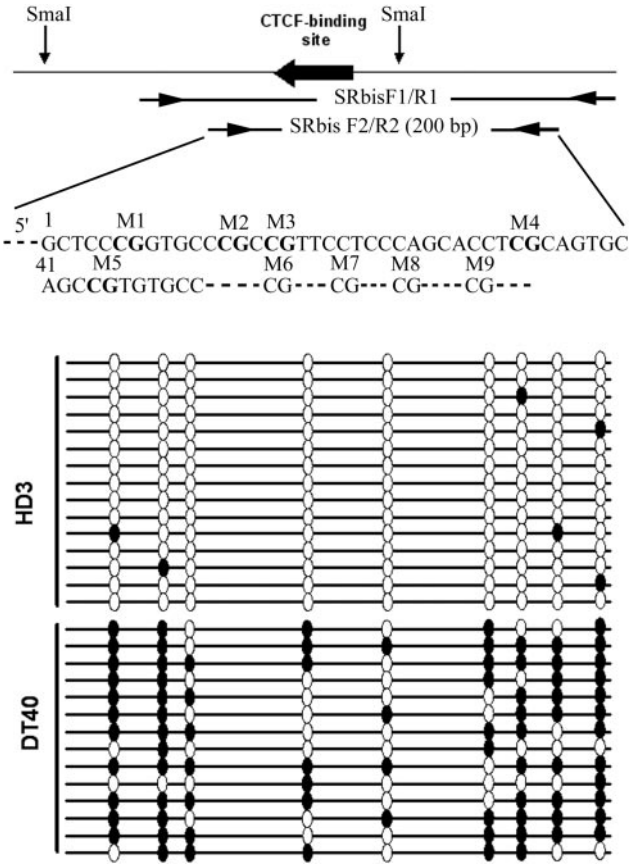

FIG. 4. Methylation status of the CTCF-binding site in erythroid and nonerythroid cell lines. Genomic DNA from HD3 and DT40 cells was bisulfite treated and amplified in a nested reaction with the primers SRbisF1 plus SRbisR1 and SRbisF2 plus SRbisR2 (see the scheme at the top of the figure). The amplified fragment covered a 200-bp region, which included the five CpG dinucleotides of the CTCF-binding site plus four downstream CpGs (labeled in boldface type). The amplified DNA fragment was cloned and sequenced as described in Materials and Methods. The results of bisulfite sequencing (analysis of 14 clones in each case) are shown at the bottom of the figure. Methylated and unmethylated CpGs are shown as filled or open circles, respectively.

next set of experiments, a nuclear extract of COS-1 cells overexpressing the chicken CTCF gene from a transfected construct controlled by the simian virus 40 promoter-enhancer (24) was used in EMSA experiments, and a shifted band was observed in approximately the same position (Fig. 2C); the amount of material in the shifted band was significantly larger, in accordance with the higher concentration of CTCF (Fig. 2B).

To verify that CTCF binds exactly to the recognition sequence predicted by computer-assisted analysis, we repeated the EMSA experiments using a 50-bp DNA fragment containing the novel CTCF-binding site, with essentially the same results. Although two retarded bands were observed (Fig. 3), only one of them (the major one) was efficiently competed by an FII double-stranded oligonucleotide (Fig. 3, lane 3 from the left) and thus represented the CTCF-binding site. In the next set of experiments, the influence of CpG methylation of the recognition site on CTCF binding was studied. As shown in Fig. 3, bottom, the recognition site contained five CpGs, of which only one (CpG 3) was present also in the FII CTCF recognition site. We found that the 50-bp DNA fragment with all five CpGs methylated completely lost the ability to bind CTCF (Fig. 3, lane FM), as expected, based on previously published data (3, 16, 17, 29). Double-stranded oligonucleotides methylated at individual positions (1 to 5) were then synthesized and tested in band-shift experiments. Methylation of individual CpGs at positions 1 or 2 (Fig. 3, lanes M1 and M2) did not influence CTCF binding, as concluded from the presence of the major retarded band; in contrast, methylation at position 3 (Fig. 3, lane M3) abolished binding completely. Methylation at positions 4 and 5 (Fig. 3, lanes M4 and M5) partially suppressed CTCF binding, suggesting that the positions of methylated CpGs are critical for interference with CTCF sequence recognition. Thus, CTCF binds in vitro to the 50-bp recognition site present within the 200-bp DNA fragment of the CpG island under study and the binding is methylation sensitive; methylation of a single ("conservative") CpG also present within the FII recognition site (CpG 3) is sufficient to completely prevent binding of CTCF.

Analysis of endogenous methylation patterns of the CTCF-binding site in the upstream CpG island of the chicken domain of α-globin genes in HD3 and DT40 cells. Since, from the results shown above, differential DNA methylation seems to play an epigenetic role in CTCF binding and regulatory properties of the CpG island, we decided to address the endogenous DNA methylation status in an erythroid (HD3 cells) and

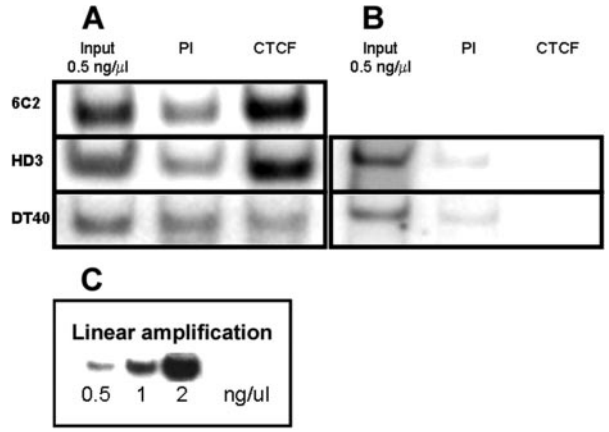

FIG. 5. In vivo interaction of CTCF with the recognition site within the chicken α-globin gene domain upstream CpG island. DNA was recovered from the immunoprecipitate after ChIP using chicken CTCF-specific antibodies (44) or preimmune serum (PI). (A) PCR amplification of the 200-bp region with the CTCF-binding site using DNA from ChIP with PI or with CTCF-specific antibodies (CTCF) from 6C2, HD3, and DT40 cells. (B) PCR amplification of the β^A/ε enhancer using DNA from ChIP with PI or with CTCF from HD3 and DT40 cells. (C) Linear dependence of PCR product on the quantity of template used. All data are representative of two independent experiments and at least three independent PCR amplifications in the linear range.

nonerythroid (DT40 cells) context. We surveyed individual CpG methylation by bisulfite genomic sequencing and methylation-specific PCR amplification; single PCR fragments of bisulfite-modified DNA were subcloned and analyzed by sequencing. Consistent with our previous data and predictions, the DNA sequences surrounding the CTCF-binding site were unmethylated in erythroid HD3 cells (Fig. 4). In contrast, genomic DNA from the lymphoid DT40 cell line was hypermethylated over the entire DNA fragment (Fig. 4) with no apparent preferential CpG methylation.

In summary, the DNA fragment which includes the CTCF motif was methylated in nonerythroid cells, interfering with CTCF binding to its recognition sequence and probably modulating, in this way, the activity of the CpG island (see below).

CTCF is bound in vivo to the recognition site. To find out if CTCF is associated in vivo with the recognition site identified in the previous experiments, ChIP with CTCF-specific antibodies was performed comparing chromatin from different erythroid and nonerythroid cells. The results (Fig. 5) showed that CTCF was clearly bound to the DNA fragment in both preerythroblasts (6C2 cells) and erythroblasts (HD3 cells). In contrast, CTCF was unable to recognize its interacting sequence in nonerythroid DT40 cells, consistent with the observed DNA methylation status of the CpG island (33). The significance of this observation will be referred to below (see Discussion). It is important to underline that CTCF is expressed in DT40 cells, as in our previous study it was shown that in these cells it interacts in vivo with one of the upstream enhancer-blocking elements of the domain of α-globin genes (44). To confirm the specificity of the ChIP assay, the DNA fragments pelleted by CTCF-specific antibodies were used as a template to amplify a fragment of the chicken β^A/ε enhancer; no amplification products were detected, in accordance with previous data that CTCF does not bind this region (39, 44).

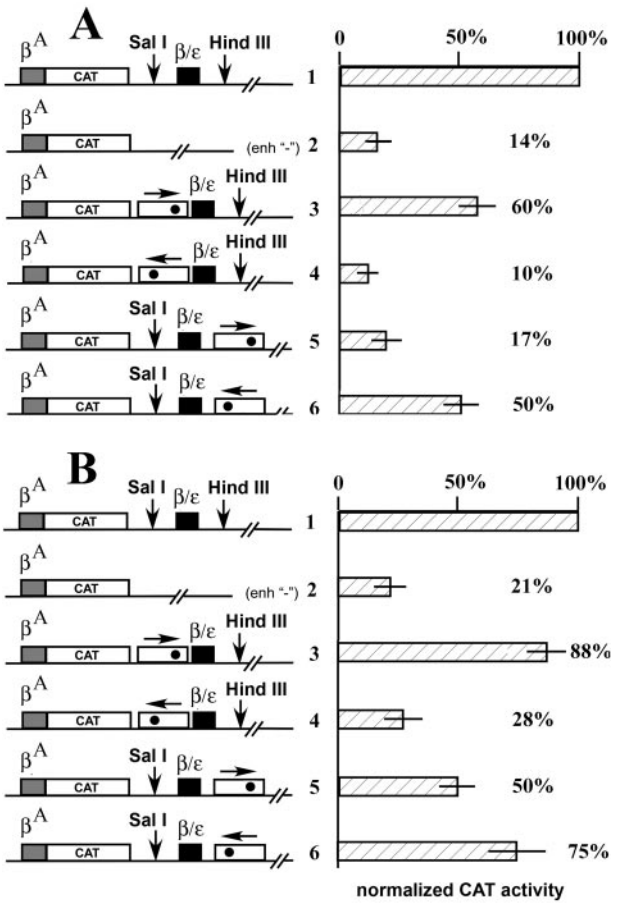

FIG. 6. Analysis of enhancer-blocking and silencing activity of the 200-bp CTCF-binding fragment by transient transfection of linearized (A) or circular (B) plasmids into HD3 cells. Constructs are shown on the left and CAT activity is shown on the right. The chicken adult β-globin promoter (gray boxes) and a minimal β^A/ε enhancer (black boxes) were used to control expression of the reporter CAT gene (construct 1). As a negative control, the same construct with a deleted β^A/ε enhancer (construct 2) was used. The 200-bp DNA fragment (white boxes) was cloned in the SalI or HindIII sites of pAcatE in different orientations (indicated by arrows showing the direction of α-globin gene transcription). The filled circle inside the 200-bp fragment indicates the approximate position of the CTCF-binding site. CAT activity was normalized considering the efficiency of transfection. Error bars show the standard error of the mean (SEM) from three independent experiments.

The 200-bp DNA fragment possesses a CTCF-dependent silencer activity but not an enhancer-blocking activity. To test the functional activity of the DNA fragment containing the identified CTCF-binding site, the transient assay for enhancer-blocking activity (37) was used. The 200-bp DNA fragment was cloned into pAcatE in both orientations upstream (between) or downstream (outside) relative to the enhancer (Fig. 6A). To avoid bidirectional action of the enhancer, all constructs were linearized and transiently transfected into HD3 cells (37). The original pAcatE (construct 1) and the enhancerless (construct 2) vectors were also transfected in each set of experiments to calibrate the system (Fig. 6A). A significant reduction in reporter gene activity (relative to pAcatE) was seen when the 200-bp DNA fragment was located between the gene block and

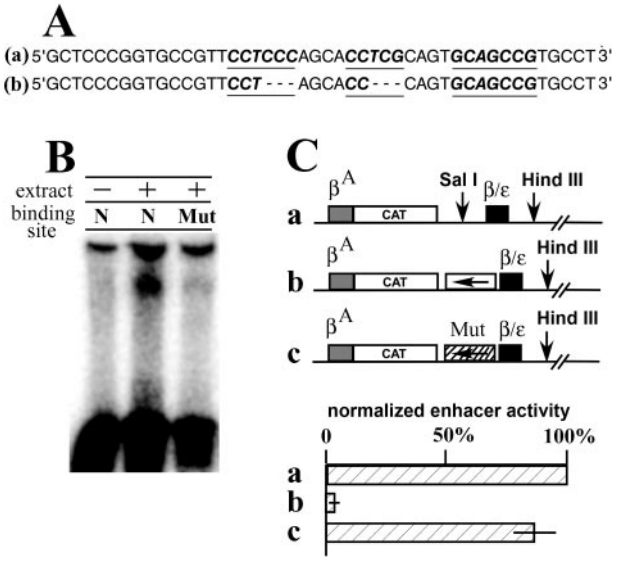

FIG. 7. Dependence of the silencing activity of the 200-bp fragment on CTCF binding. (A) Putative CTCF-binding sequence with regions believed to be critical for binding underlined (a); mutated fragment with two of the three regions partially deleted (b). (B) CTCF binding to the 200-bp native (N) and mutated (Mut) fragment in a gel retardation assay. (C) Silencing activity of the native 200-bp DNA fragment (construct b) and of the fragment with a mutated CTCF-binding site (construct c). The β^A/ε enhancer activity, calculated as the difference between the activities displayed by the pAcatE construct (construct a) and by the construct with a deleted β^A/ε enhancer, was taken as 100%. All the constructs were linearized before transfection into HD3 cells. Error bars show the SEM of three independent experiments.

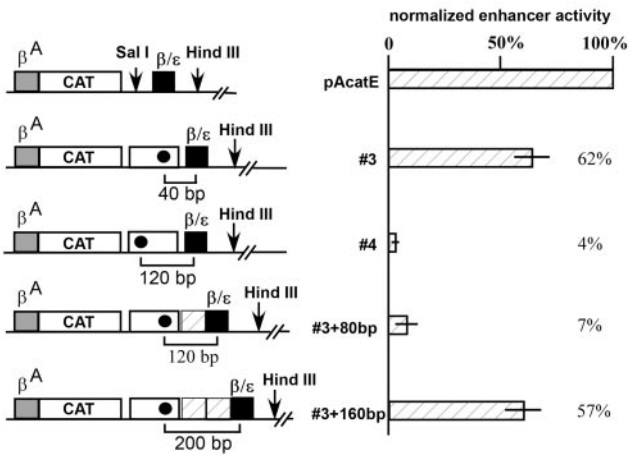

FIG. 8. Dependence of the silencing activity on the distance between the CTCF-binding site and the enhancer. The constructs are shown on the left; the 200-bp SmaI fragment under study is shown by the open box containing a filled circle showing the approximate position of the CTCF-binding site. The DNA fragments inserted between the β^A/ε enhancer and the 200-bp fragment are shown by hatched boxes. Enhancer activity is presented as shown in Fig. 7.

the enhancer in the reverse orientation (Fig. 6A, construct 4) or downstream to the enhancer in the direct orientation (Fig. 6A, construct 5). In other cases, a less-prominent reduction of CAT gene activity was observed. Taken together, these results do not corroborate the idea that the 200-bp DNA fragment harbors a positional enhancer-blocking element; on the contrary, they are indicative of the presence of a silencer element within this fragment. This conclusion was further supported by the results obtained upon transfection of circular constructs (Fig. 6B).

To verify inherence of the newly defined CTCF binding site for the silencing effect, a 200-bp SmaI fragment bearing a mutated binding site was constructed (Fig. 7A). This fragment did not bind CTCF, as shown by gel retardation experiments using nuclear extract from HD3 cells (Fig. 7B); at the same time, silencing activity was abolished, as shown by transient transfection experiments (Fig. 7C). Thus, the activity of the silencer present within the fragment depends on CTCF binding.

The distance between the CTCF binding site and the enhancer is crucial for silencing activity. In transient transfection experiments, maximal silencing activity was observed for constructs 4 and 5 (Fig. 6A) where the 200-bp DNA fragment was cloned either in reverse orientation upstream to the enhancer or in direct orientation downstream to the enhancer. The only common feature of these two constructs was that the CTCF-binding site asymmetrically located within the 200-bp SmaI fragment was placed at the same distance, about 120 bp from the enhancer. We propose that the distance between the CTCF-binding site and the enhancer may be of importance for the formation of a silencing complex involving CTCF and proteins bound to the enhancer. To test this supposition, we inserted an 80-bp fragment of pUC DNA in the XhoI site between the enhancer and the 200-bp SmaI fragment cloned upstream to the enhancer in the direct orientation (e.g., Fig. 6A, construct 3, which showed very weak silencing activity). The insertion of an 80-bp DNA fragment placed the CTCF-binding site at ~120 bp from the enhancer. When this new construct was tested in transient transfection experiments, a significant (15 fold compared to construct 3) increase of silencing activity was observed (Fig. 8) or about the same as that of original constructs 4 and 5 (Fig. 6A). Insertion of a second 80-bp DNA fragment between the enhancer and the 200-bp SmaI fragment, which placed the CTCF-binding site at ~200 bp from the enhancer, resulted in a sharp (eightfold) decrease in silencing activity (Fig. 8). Apparently, location of the CTCF-binding site at a distance of ~120 bp from the target β^A/ε enhancer is indeed crucial for the manifestation of maximal silencing activity.

The promoter of the ggPRX gene is located at a distance of 120 bp from the CTCF-dependent silencer. To identify the possible natural target for the CTCF-dependent silencer, we mapped the promoter of the chicken *ggPRX* gene. As the promoter of the human homolog of this gene was already mapped (46), we analyzed the potential homology between human and chicken sequences within this area (Fig. 9A), and found a region showing a certain degree of sequence similarity to the human *-14* gene promoter within the limits of the CpG island of the chicken α-globin gene domain, 120 bp from the CTCF-binding site characterized in the present work. This 110-bp DNA fragment was PCR amplified and cloned into the promoter site of the pCAT3 basic vector (Promega), and this construct was transfected into erythroid HD3 cells. CAT activity was determined 48 h after transfection. A similar con-

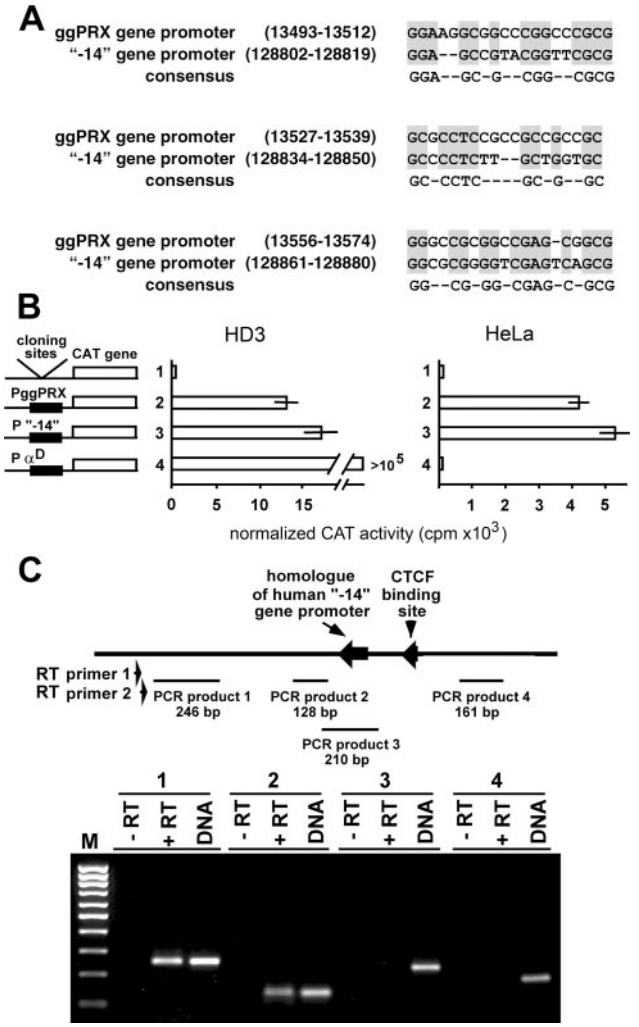

FIG. 9. Identification of the chicken *ggPRX* gene promoter. (A) Alignment of the nucleotide sequences of the human *-14* gene promoter region (numbers correspond to GenBank sequence AE006462) and of the CpG island from the upstream region of the chicken α-globin domain (numbers correspond to GenBank sequence AF098919). The regions sharing sequence identity are shaded. (B) Transient transfection experiments in HD3 cells (left) and HeLa cells (right); the constructs are shown on the far left and CAT activity is shown on the right. As a negative control, the promoterless pCAT3 basic vector (Promega) was used, and a construct with the erythroid cell-specific chicken α^D-globin promoter driving CAT gene expression was used as a positive control in HD3 cells and as a negative control in HeLa cells. CAT activity was normalized, considering the efficiency of transfection. Data are the average of three independent experiments, and error bars show the SEM. (C) RT-PCR analysis of *ggPRX* gene transcription in HD3 cells. (Top) Positions of the regions examined on the map of the upstream part of the chicken α-globin gene domain. (Bottom) Amplification of test regions 1 to 4. Lanes marked "−RT" were loaded with products of direct PCR amplification of RNA (without RT polymerase); lanes marked "DNA" were loaded with products of amplification of 100 ng of chicken genomic DNA.

struct containing the homologous 110-bp DNA fragment with the human *-14* gene promoter was used as a positive control. The results shown in Fig. 9B demonstrate that the chicken DNA fragment possesses about the same promoter activity as the previously described promoter of the human *-14* gene. Similar results were obtained when the activity of both promoters was tested in chicken lymphoid DT40 cells (data not shown). Both human and chicken promoters were also found to be active in HeLa cells (Fig. 9B). In contrast, the chicken α^D-globin gene promoter was not active in HeLa cells, although it was extremely active in chicken erythroid cells (Fig. 9B). To verify that the identified promoter does direct the transcription of the *ggPRX* gene in vivo, an RT-PCR assay with primers scattered along the area was carried out. The results (Fig. 9C) demonstrate that regions located downstream to the promoter (test regions 1 and 2) were transcribed in the direction of *ggPRX* gene transcription, while the regions encompassing promoter (test region 3) and located upstream to the promoter (test region 4) were not transcribed in this direction. Thus, the promoter identified directs the transcription of the *ggPRX* gene. Taking into consideration the results of our transient transfection experiments, we conclude that the activity of this promoter is not tissue or species specific, in agreement with the previously published supposition that *ggPRX* is a housekeeping gene that is highly conserved through the evolution of vertebrates (13, 42, 46).

DISCUSSION

In this paper, we describe a novel CTCF-dependent silencer located within the upstream CpG island of the chicken α-globin gene domain. This CpG island contains a considerable number of other important functional elements including a replication origin (35, 45), a site of DNA loop anchorage to the nuclear matrix (11, 36), a promoter of a housekeeping gene (42, 46), a weak enhancer (34), and binding sites for different transcription factors (8, 40). The CTCF-dependent silencer identified in the present study is most likely involved in regulation of the activity in erythroid cells of the evolutionarily conserved housekeeping *ggPRX* gene. We found that the distance (relatively short) between the CTCF-binding site and the enhancer is crucial for the silencer's functioning; the promoter of the *ggPRX* gene is 120 bp downstream to the CTCF-dependent silencer. At least in the model experiments described here, the silencer had maximal activity when placed at this distance from the enhancer. The promoter of the *ggPRX* gene can be considered a promoter-enhancer unit, as it works like the promoter of a human analog of the *ggPRX* gene, without the inclusion of an additional enhancer.

The *ggPRX* gene and its analogs in other vertebrates (21, 46) are located at a short distance upstream to the α-globin gene domain and are transcribed in the opposite direction. Interestingly, the regulatory systems controlling expression of these genes overlap the regulatory domains of the α-globin gene cluster. In this context, it is interesting that in nonerythroid chicken cells, the area including the CTCF-binding site described here is extensively methylated (33), as confirmed in the present study with bisulfite sequencing. In lymphoid DT40 cells, the CTCF recognition site was shown to be hypermethylated, and it is known from the literature (3, 16, 17) and shown here for the particular recognition sequence studied that methylation interferes with CTCF binding. Interestingly and contrary to other divergent CTCF-binding sites, the newly defined motif possesses five CpG dinucleotides that are targets for

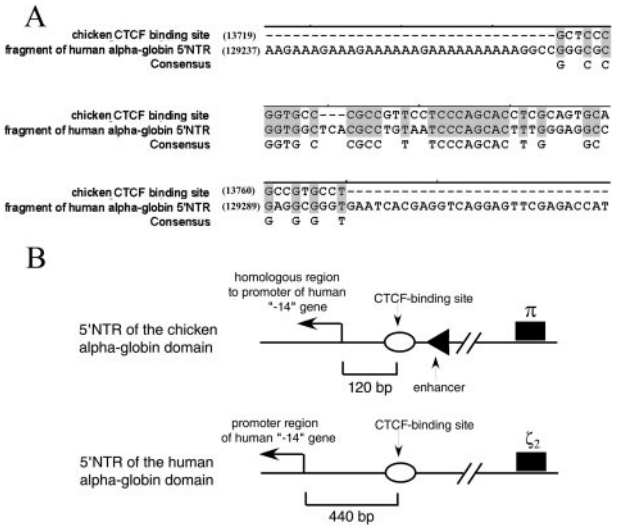

FIG. 10. Evolutionary conservation of the mutual location of regulatory elements in the upstream regions of α-globin gene domains. (A) Sequence alignment of the putative CTCF-binding site (50 bp) from the CpG island of the upstream region of the chicken α-globin domain, with part of the upstream region of the human α-globin domain (numbers correspond to GenBank sequenceAE006462). The regions of sequence identity are shaded. (B) Upstream regions of the chicken and human α-globin gene domains. Embryonic globin genes are shown by black rectangles, CTCF-binding sites are shown by open ovals, and the non-tissue-specific enhancer from the upstream region of the chicken α-globin gene domain is shown by the filled arrowhead. The promoter region of the human *-14* gene and the homologous region from the upstream area of the chicken α-globin gene domain are shown by arrows indicating the direction of *-14* gene transcription.

methylation (Fig. 1B and 3). However, we demonstrated here that methylation of only one of these CpGs, namely, that which is conserved between the CTCF-binding site under study and FII, the best-characterized CTCF-binding site from the upstream insulator of the chicken β-globin gene domain (2), is sufficient to prevent CTCF binding. Methylation of some other CpG sites within the CTCF-binding motif identified in this study partially suppressed CTCF binding. Thus, the silencer element described in this paper should be active only in erythroid cells where the CTCF recognition site is not methylated, in contrast to lymphoid and possibly other nonerythroid cells (33). Indeed, we demonstrated by ChIP that CTCF was clearly associated with this silencer in erythroid but not in lymphoid cells where the recognition site is methylated (33). Activation of the silencer in erythroid cells is likely to stabilize the level of *ggPRX* gene expression in a situation when the promoter of this gene may be influenced by positive *cis*-regulatory signals that activate α-globin gene transcription, and the level of histone acetylation within an extended chromatin domain including the *ggPRX* gene promoter increases (1), while hypermethylation of the area adjacent to the *ggPRX* promoter is lost (33). Taking into consideration the evolutional conservation of the mutual positions of α-globin gene domains and *ggPRX*, *-14*, and *mProx1* housekeeping genes in different vertebrates (13, 21, 42, 46), it was of interest to check if the CTCF-dependent silencer was also present in the vicinity of the promoters of these genes. Computer analysis of the upstream area of the human α-globin gene domain allowed us to identify a potential silencer sharing 70% sequence identity with the chicken silencer element described here and located at a distance of 440 bp upstream to the promoter of the *-14* gene (Fig. 10A). Thus, an evolutionary conservation of the mutual location of regulatory elements in the upstream areas of the vertebrate α-globin gene domains is likely to exist (Fig. 10B).

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REFERENCES

1. **Anguita, E., C. A. Johnson, W. G. Wood, B. M. Turner, and D. R. Higgs.** 2001. Identification of a conserved erythroid specific domain of histone acetylation across the alpha-globin gene cluster. Proc. Natl. Acad. Sci. USA **98:**12114–12119.
2. **Bell, A. C., A. G. West, and G. Felsenfeld.** 1999. The protein CTCF is required for the enhancer-blocking activity of vertebrate insulators. Cell **98:**387–396.
3. **Bell, A. C., and G. Felsenfeld.** 2000. Methylation of a CTCF-dependent boundary controls imprinted expression of the Igf2 gene. Nature **405:**482–485.
4. **Beug, H., G. Doederlein, C. Freudenstrein, and T. Graf.** 1979. Erythroblast cell lines transformed by a temperature sensitive mutant of avian erythroblastosis virus. A model system to study erythroid differentiation in vitro. J. Cell Physiol. **1:**195–207.
5. **Beug, H., S. Palmieri, C. Freudenstein, H. Zentgraf, and T. Graf.** 1982. Hormone-dependent terminal differentiation in vitro of chicken erythroleukemia cells transformed by ts mutants of avian erythroblastosis virus. Cell **28:**907–919.
6. **Chung, J. H., A. C. Bell, and G. Felsenfeld.** 1997. Characterization of the chicken beta-globin insulator. Proc. Natl. Acad. Sci. USA **94:**575–580.
7. **Craddock, C. F., P. Vyas, J. A. Sharpe, H. Ayyub, W. G. Wood, and D. R. Higgs.** 1995. Contrasting effects of alpha and beta globin regulatory elements on chromatin structure may be related to their different chromosomal environments. EMBO J. **14:**1718–1726.
8. **De Moura Gallo, C. V., J. Y. S. Vassetzky, F. Recillas Targa, G. P. Georgiev, K. Scherrer, and S. V. Razin.** 1991. The presence of sequence-specific protein binding site correlate with replication activity and matrix binding in a 1.7 kb-long DNA fragment of the chicken α-globin gene domain. Biochem. Biophys. Res. Commun. **179:**512–519.
9. **Dillon, N., and P. Sabbattini.** 2000. Functional gene expression domains: defining the functional unit of eukaryotic gene regulation. Bioessays **22:**657–665.
10. **Docquier, F., D. Farrar, V. D'Arcy, I. Chernukhin, A. F. Robinson, D. Loukinov, S. Vatolin, S. Pack, A. Mackay, R. A. Harris, H. Dorricott, M. J. O'Hare, V. Lobanenkov, and E. Klenova.** 2005. Heightened expression of CTCF in breast cancer cells is associated with resistance to apoptosis. Cancer Res. **65:**5112–5122.
11. **Farache, G., S. V. Razin, F. Recillas Targa, and K. Scherrer.** 1990. Organization of the 3′-boundary of the chicken α-globin gene domain and characterization of a CR1-specific protein binding site. Nucleic Acids Res. **18:**401–409.
12. **Filippova, G. N., S. Fagerlie, E. M. Klenova, C. Myers, Y. Dehner, G. Goodwin, P. E. Neiman, S. J. Collins, and V. V. Lobanenkov.** 1996. An exceptionally conserved transcriptional repressor, CTCF, employs different combinations of zinc fingers to bind diverged promoter sequences of avian and mammalian c-*myc* oncogenes. Mol. Cell. Biol. **16:**2802–2813.
13. **Flint, J., C. Tufarelli, J. Peden, K. Clark, R. J. Daniels, R. Hardison, W. Miller, S. Philipsen, K. C. Tan-Un, T. McMorrow, J. Frampton, B. P. Alter, A. M. Frischauf, and D. R. Higgs.** 2001. Comparative genome analysis delimits a chromosomal domain and identifies key regulatory elements in the alpha globin cluster. Hum. Mol. Genet. **10:**371–382.

14. **Fried, M., and D. M. Crothers.** 1981. Equilibria and kinetics of lac repressor-operator interactions by polyacrylamide gel electrophoresis. Nucleic Acids Res. **9:**6505–6524.
15. **Groudine, M., and H. Weintraub.** 1982. Propagation of globin DNaase I-hypersensitive sites in absence of factors required for induction: a possible mechanism for determination. Cell **30:**131–139.
16. **Hark, A. T., C. J. Schoenherr, D. J. Katz, R. S. Ingram, J. M. Levorse, and S. M. Tilghman.** 2000. CTCF mediates methylation-sensitive enhancer-blocking activity at the H19/Igf2 locus. Nature **405:**486–489.
17. **Holmgren, C., C. Kanduri, G. Dell, A. Ward, R. Mukhopadhya, M. Kanduri, V. Lobanenkov, and R. Ohlsson.** 2001. CpG methylation regulates the Igf2/H19 insulator. Curr. Biol. **11:**1128–1130.
18. **Kalandadze, A. G., S. A. Bushara, J. Y. S. Vassetzky, and S. V. Razin.** 1990. Characterization of DNA pattern in the site of permanent attachment to the nuclear matrix located in the vicinity of replication origin. Biochem. Biophys. Res. Commun. **168:**9–15.
19. **Kanduri, C., V. Pant, D. Loukinov, E. Pugacheva, C. F. Qi, A. Wolffe, R. Ohlsson, and V. V. Lobanenkov.** 2000. Functional association of CTCF with the insulator upstream of the H19 gene is parent of origin-specific and methylation-sensitive. Curr. Biol. **10:**853–856.
20. **Kemper, B., P. D. Jackson, and G. Felsenfeld.** 1987. Protein-binding sites within the 5′ DNase I-hypersensitive region of the chicken α^D-globin gene. Mol. Cell. Biol. **7:**2059–2069.
21. **Kielman, M. F., R. Smits, I. Hof, and L. F. Bernini.** 1996. Characterization and comparison of the human and mouse Dist1/α-globin complex reveals a tightly packed multiple gene cluster containing differentially expressed transcription units. Genomics **32:**341–351.
22. **Kielman, M. F., R. Smits, T. S. Devi, R. Fodde, and L. F. Bernini.** 1993. Homology of a 130-kb region enclosing the α-globin gene cluster, the α-locus controlling region, the two non-globin genes in human and mouse. Mamm. Genome **4:**314–323.
23. **Klenova, E. M., R. H. Nicolas, H. F. Paterson, A. F. Carne, C. M. Heath, G. H. Goodwin, P. E. Neiman, and V. V. Lobanenkov.** 1993. CTCF, a conserved nuclear factor required for optimal transcriptional activity of the chicken c-*myc* gene, is an 11-Zn-finger protein differentially expressed in multiple forms. Mol. Cell. Biol. **13:**7612–7624.
24. **Klenova, E. M., R. H. Nicolas, S. U., A. F. Carne, R. E. Lee, V. V. Lobanenkov, and G. H. Goodwin.** 1997. Molecular weight abnormalities of the CTCF transcription factor: CTCF migrates aberrantly in SDS-PAGE and the size of the expressed protein is affected by the UTRs and sequences within the coding region of the CTCF gene. Nucleic Acids Res. **25:**466–474.
25. **Knezetic, J. A., and G. Felsenfeld.** 1989. Identification and characterization of a chicken α-globin enhancer. Mol. Cell. Biol. **9:**893–901.
26. **Lobanenkov, V. V., R. H. Nicolas, V. V. Adler, H. Paterson, E. M. Klenova, A. V. Polotskaja, and G. H. Goodwin.** 1990. A novel sequence-specific DNA binding protein which interacts with three regularly spaced direct repeats of the CCCTC-motif in the 5′-flanking sequence of the chicken c-myc gene. Oncogene **5:**1743–1753.
27. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
28. **Moon, H., G. Filippova, D. Loukinov, E. Pugacheva, Q. Chen, S. T. Smith, A. Munhall, B. Grewe, M. Bartkuhn, R. Arnold, L. J. Burke, R. Renkawitz-Pohl, R. Ohlsson, J. Zhou, R. Renkawitz, and V. Lobanenkov.** 2005. CTCF is conserved from Drosophila to humans and confers enhancer blocking of the Fab-8 insulator. EMBO Rep. **6:**165–170.
29. **Mukhopadhyay, R., W. Yu, J. Whitehead, J. Xu, M. Lezcano, S. Pack, C. Kanduri, M. Kanduri, V. Ginjala, A. Vostrov, W. Quitschke, I. Chernukhin, E. Klenova, V. Lobanenkov, and R. Ohlsson.** 2004. The binding sites for the chromatin insulator protein CTCF map to DNA methylation-free domains genome-wide. Genome Res. **14:**1594–1602.
30. **Ohlsson, R., R. Renkawitz, and V. Lobanenkov.** 2001. CTCF is a uniquely versatile transcription regulator linked to epigenetics and disease. Trends Genet. **17:**520–527.
31. **Razin, S. V., C. V. De Moura Gallo, and K. Scherrer.** 1994. Characterization of the chromatin structure in the upstream area of the chicken α-globin gene domain. Mol. Gen. Genet. **242:**649–652.
32. **Razin, S. V., C. M. Farrell, and F. Recillas-Targa.** 2003. Genomic domains and regulatory elements operating at the domain level. Int. Rev. Cytol. **226:**63–125.
33. **Razin, S. V., E. S. Ioudinkova, and K. Scherrer.** 2000. Extensive methylation of a part of the CpG island located 3.0–4.5 kbp upstream to the chicken alpha-globin gene cluster may contribute to silencing the globin genes in non-erythroid cells. J. Mol. Biol. **209:**845–852.
34. **Razin, S. V., J. Y. S. Vassetzky, A. I. Kvartskhava, N. F. Grinenko, and G. P. Georgiev.** 1991. Transcriptional enhancer in the vicinity of replication origin within the 5′ region of the chicken α-globin gene domain. J. Mol. Biol. **217:**595–598.
35. **Razin, S. V., M. G. Kekelidze, E. M. Lukanidin, K. Scherrer, and G. P. Georgiev.** 1986. Replication origins are attached to the nuclear skeleton. Nucleic Acids Res. **14:**8189–8207.
36. **Razin, S. V., P. Petrov, and R. Hancock.** 1991. Precise localization of the α-globin gene cluster within one of the 20- to 300-kilobase DNA fragment released by cleavage of chicken chromosomal DNA at topoisomerase II site in vivo: evidence that the fragment are DNA loops or domains. Proc. Natl. Acad. Sci. USA **88:**8515–8519.
37. **Recillas-Targa, F., A. C. Bell, and G. Felsenfeld.** 1999. Positional enhancer-blocking activity of the chicken beta-globin insulator in transiently transfected cells. Proc. Natl. Acad. Sci. USA **96:**14354–14359.
38. **Recillas Targa, F., C. V. De Moura Gallo, M. Huesca, K. Scherrer, and L. Marcaud.** 1993. Silencer and enhancer elements located at the 3′-side of the chicken and duck α-globin-encoding gene domains. Gene **129:**229–237.
39. **Recillas-Targa, F., M. J. Pikaart, B. Burgess-Beusse, A. C. Bell, M. D. Litt, A. G. West, M. Gaszner, and G. Felsenfeld.** 2002. Position-effect protection and enhancer blocking by the chicken beta-globin insulator are separable activities. Proc. Natl. Acad. Sci. USA **99:**6883–6888.
40. **Recillas-Targa, F., S. V. Razin, C. V. de Moura Gallo, and K. Scherrer.** 1994. Excision close to matrix attachment regions of the entire chicken a-globin gene domain by nuclease S1 and characterization of the framing structures. Proc. Natl. Acad. Sci. USA **91:**4422–4426.
41. **Reitman, M., and G. Felsenfeld.** 1988. Mutational analysis of the chicken beta-globin enhancer reveals two positive-acting domains. Proc. Natl. Acad. Sci. USA **85:**6267–6271.
42. **Sjakste, N., O. V. Iarovaia, S. V. Razin, G. Linares-Cruz, T. Sjakste, V. Le Gac, Z. Zhao, and K. Scherrer.** 2000. A novel gene is transcribed in the chicken alpha-globin gene domain in the direction opposite to the globin genes. Mol. Gen. Genet. **262:**1012–1021.
43. **Stalder, J., A. Larsen, J. D. Engel, M. Dolan, M. Groudine, and H. Weintraub.** 1980. Tissue-specific DNA cleavages in the globin chromatin domain introduced by DNAase I. Cell **20:**451–460.
44. **Valadez-Graham, V., S. V. Razin, and F. Recillas-Targa.** 2004. CTCF-dependent enhancer blockers at the upstream region of the chicken alpha-globin gene domain. Nucleic Acids Res. **32:**1354–1362.
45. **Verbovaia, L., and S. V. Razin.** 1995. Analysis of the replication direction through the domain of a-globin-encoding genes. Gene **166:**255–259.
46. **Vyas, P., M. A. Vickers, D. J. Picketts, and D. R. Higgs.** 1995. Conservation of position and sequence of a novel, widely expressed gene containing the major human alpha-globin regulatory element. Genomics **29:**679–689.
47. **Vyas, P., M. A. Vickers, D. L. Simmons, H. Ayyub, C. F. Craddock, and D. R. Higgs.** 1992. cis-acting sequences regulating expression of the human alpha-globin cluster lie within constitutively open chromatin. Cell **69:**781–793.