MOLECULAR AND CELLULAR BIOLOGY, June 2006, p. 4368–4377 Vol. 26, No. 11
0270-7306/06/$08.00+0 doi:10.1128/MCB.02216-05

Alterations in Expression and Chromatin Configuration of the Alpha Hemoglobin-Stabilizing Protein Gene in Erythroid Krüppel-Like Factor-Deficient Mice†

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Received 16 November 2005/Returned for modification 6 December 2005/Accepted 6 March 2006

Erythroid Krüppel-like factor (EKLF) is an erythroid zinc finger protein identified by its interaction with a CACCC sequence in the β-globin promoter, where it establishes local chromatin structure permitting β-globin gene transcription. We sought to identify other EKLF target genes and determine the chromatin status of these genes in the presence and absence of EKLF. We identified alpha hemoglobin-stabilizing protein (AHSP) by subtractive hybridization and demonstrated a 95 to 99.9% reduction in AHSP mRNA and the absence of AHSP in EKLF-deficient cells. Chromatin at the AHSP promoter from EKLF-deficient cells lacked a DNase I hypersensitive site and exhibited histone hypoacetylation across the locus compared to hyperacetylation of wild-type chromatin. Wild-type chromatin demonstrated a peak of EKLF binding over a promoter region CACCC box that differs from the EKLF consensus by a nucleotide. In mobility shift assays, the AHSP promoter CACCC site bound EKLF in a manner comparable to the β-globin promoter CACCC site, indicating a broader recognition sequence for the EKLF consensus binding site. The AHSP promoter was transactivated by EKLF in K562 cells, which lack EKLF. These results support the hypothesis that EKLF acts as a transcription factor and a chromatin modulator for the AHSP and β-globin genes and indicate that EKLF may play similar roles for other erythroid genes.

EKLF (erythroid Krüppel-like factor, or KLF1) is one of several CACCC-binding regulatory proteins active in erythroid cells (2, 3, 5, 6, 17, 48, 74). It is the founding member of the mammalian Krüppel subfamily of transcription factors with 3 C_2H_2-type zinc fingers at the COOH terminus (7, 8, 35, 55). Its roles include β-globin gene activation, participation in the switch to adult β-globin, and coordination of erythroid cell proliferation and hemoglobinization (11, 18, 31, 39, 56, 65, 69). EKLF binds to the consensus sequence CCNCNCCCN in the β-globin gene promoter proximal CACCC box (22, 48), where it establishes local chromatin structure, as evidenced by DNase I hypersensitive site (HS) formation and chromatin immunoprecipitation (ChIP) studies, and directs high-level β-globin transcription. Mutations of this consensus sequence in the β-globin promoter lead to the phenotype of β-thalassemia (40, 52, 53).

The β-thalassemia syndromes are marked by clinical heterogeneity, and numerous modifier genes have been proposed (66). Recently, α-hemoglobin stabilizing protein (AHSP, also known as erythroid differentiation-related factor and erythroid-associated factor) has been proposed as a β-thalassemia modifier gene (19, 37, 38, 46, 68). AHSP binds and stabilizes free α-hemoglobin, inhibiting the production of reactive oxygen species from α-hemoglobin and preventing the precipitation of unstable, cytotoxic free α-globin chains. Free α-globin chains aggregate in erythroid precursors, damaging the membrane and triggering cell death. When β-thalassemic mice were bred to AHSP-deficient mice, AHSP deficiency worsened the thalassemic phenotype, leading to the suggestion that AHSP could be a modifier gene in human β-thalassemia syndromes (38).

EKLF-deficient mice die at embryonic day 14.5 (E14.5) to E15 from severe anemia due to defective definitive erythropoiesis (44, 50, 57). There is a marked decrease in β-globin mRNA and protein levels in EKLF-deficient erythroid cells. In addition, there are alterations in chromatin configuration at the β-globin gene promoter, including loss of a DNase I hypersensitive site at the proximal CACCC box as well as diminution of another HS site over 50 kb away in the β-globin locus control region (HS3) (26, 69). Large amounts of iron accumulate in the reticuloendothelial system of EKLF-deficient mice, consistent with ineffective erythropoiesis and hemolysis. These observations led to the suggestion that the fatal anemia experienced by these EKLF-deficient mice was due entirely to deficient β-globin expression.

Several observations have suggested that there are additional erythroid cell defects in EKLF-deficient mice. Fetal liver-derived circulating erythroid cells in EKLF-deficient embryos exhibit a greater degree of hemolysis than typically occurs in β-thalassemia major (50, 57). Disruption of the β^{major} and β^{minor} genes in mice leads to anemia and death, but unlike the EKLF-

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† Supplemental material for this article may be found at http://mcb.asm.org/.

TABLE 1. Primers used in quantitative RT-PCR

Gene	Primer sequence (5′–3′)	Location
AHSP	CGAGGGTTCACCCAGTCATGAACCACAATC	5′ UTR
	ATTGTGGATGAGGCGGGCTC	Exon 2
Protein 4.9	CTCCGGGTGTGGACCGCATGAGG	3′ UTR
	CAGCAGAGGAAGAGGACACTGTA	Penultimate exon
Rh30	GTTGTCTCCTAAGTGTCAGAGTGTGGAGGGCTCCC	3′ UTR
	CATCCCAAAGTATTAATAGTCTCTGTCATGAAACC	Penultimate exon
Aquaporin 1	GGTGCCCTGGCAGTGCTCATCTA	3′ UTR
	GGAGTGACTTTGGTCAGCTTGTC	Penultimate exon
p55	GTTCATTGCACCTACTGACCAGG	3′ UTR
	CTAGTTGGAGCAGCCCTGTTTGT	Penultimate exon
Protein 4.2	GGAAGTAGATTGTGACATGTTCC	3′ UTR
	GAGTTCCTGGACCAACAGCACTA	Penultimate exon
ERMAP	GCCCGATGTCAGACCACCCTGCCTG	5′ UTR
	CAGGGACTTGGTCTCTCCATATGAAC	Exon 2
β-Actin	GTGGGCCGCTCTAGGCACCA	
	CGGTTGGCCTTAGGGTTCAGGGGGG	
α-Globin	GGAAGATTGGTGGCCATGGTG	
	TGACCTGGGCAGAGCCGTGGC	

deficient embryos, erythroid morphology in these animals closely mirrors that seen in human patients with β-thalassemia major (16, 61, 63, 71). Finally, in rescue experiments with EKLF-deficient mice, overexpression of a human γ-globin transgene improved globin chain imbalance, but hemolysis persisted and survival was not improved (44, 49, 58).

We hypothesized that the expression of erythroid genes other than β-globin was also affected by EKLF deficiency, contributing to the anemia experienced by EKLF-deficient mice. Subtractive hybridization with fetal liver RNA of wild-type and EKLF-deficient mice identified several potential EKLF target genes, including AHSP. Levels of AHSP mRNA were reduced 95 to 99.9%, and AHSP was undetectable in the EKLF-deficient fetal liver. In the AHSP promoter region, chromatin from a wild-type fetal liver demonstrated a DNase I hypersensitive site that was absent in the EKLF-deficient fetal liver. ChIP analyses identified two regions of histone hyperacetylation in wild-type chromatin, one corresponding to the location of the hypersensitive site and the other in the 3′ end of the gene. In EKLF-deficient chromatin, histones across the AHSP locus were hypoacetylated. Two regions of EKLF binding were found in wild-type chromatin that corresponded to the regions of histone hyperacetylation, one at the hypersensitive site, peaking over an AHSP promoter CACCC box, and the other in the 3′ end of the gene. In mobility shift assays, the AHSP promoter CACCC box, which differs from the EKLF consensus by 1 nucleotide, bound EKLF in a manner comparable to the β-globin promoter CACCC box. Additional studies demonstrated that this nucleotide was not critical for EKLF binding. When combined with recent studies of finger one of the closely related transcription factor Sp1, these data indicate a broader recognition sequence for the EKLF consensus binding site. In K562 cells, the AHSP promoter was transactivated by EKLF. These results demonstrate that EKLF acts as a transcription factor and a chromatin modulator for both β-globin and AHSP and may perform the same function for other erythroid genes as well.

MATERIALS AND METHODS

Identification of EKLF target genes by cDNA subtraction. To identify differentially expressed genes, in wild-type and EKLF-deficient erythroid cells, we used a cDNA subtraction technique. The subtraction utilized fetal livers obtained from mice rendered null for the EKLF by gene targeting (57). EKLF-deficient embryos were initially identified by pallor and confirmed by genotyping (57). Ten micrograms of total RNA isolated from E13.5 wild-type and EKLF-deficient fetal livers was reverse transcribed with a modified oligo(dT) primer, 5′-TTTT GTACAAGCTT$_{30}$N$_1$N-3′, followed by coupling with (dC) tailing using a reverse transcriptase with terminal transferase activity (PowerScript; Clontech). Differential subtraction was performed as described by the manufacturer (PCR-Select cDNA Subtraction; BD Clontech). Nucleotide sequence analysis of differentially expressed clones was performed.

Analysis of differentially expressed genes. A dual riboprobe containing sequences for both exon 2 of the murine α-globin gene and the last exon of the gene of interest was created for use in RNase protection assay (RPA) quantitation. This riboprobe ensures that both sequences are labeled to equal specific activity, allowing direct comparison of mRNA levels of target genes and α-globin mRNA levels. Quantitative, real-time PCR confirmation of differential mRNA expression was performed using primers for individual target genes that amplify the penultimate exon to the 3′ untranslated region (UTR) or the 5′ UTR to exon 2 (to decrease amplification of potentially homologous genes and detect genomic DNA contamination) and an internal control, β-actin, and SYBR green for detection using an iCycler instrument (Bio-Rad) (Table 1). Reverse transcription (RT)-PCR was performed with *Thermus thermophilus* polymerase (BD Clontech), a polymerase with reverse transcriptase and polymerase activities. Values obtained for target gene expression were normalized to β-actin and were expressed relative to the expression in control samples. For calculations, the $2^{-\Delta\Delta C_T}$ formula was used, with $\Delta\Delta C_T = (C_{T,\ \text{target}} - C_{T,\ \text{GAPDH}})_{\text{experimental sample}} - (C_{T,\ \text{target}} - C_{T,\ \text{GAPDH}})_{\text{control samples}}$, where C_T is cycle threshold.

Western blot analysis was performed using wild-type and EKLF-deficient E13.5 total fetal liver proteins. Blots were probed with either a rat anti-mouse AHSP monoclonal antibody (37) or an anti-actin antibody (sc-1616; Santa Cruz).

TABLE 2. Primers used for ChIP analysis of the murine AHSP locus

Primer no.	Location in AF485327 (bp)	Sequence (5′–3′)
1	187	GTGGCTCTGCTCTCCTCTCTATAAAG
	287	GCTTGTTTGAAAAGCCAGAAAGC
2	975	CACAGGTTGTAACTGTGAGATCTTGG
	1088	TCTCACCCTGACTCTATCTGGTATGTAGTAG
3	1303	AAAGGATACTTATGTGGGTCCAGG
	1581	CCCTCCTCTGGTGTGTCTGAG
4	2022	CCTTTGCCTCCTTACCCAGC
	2126	GGTAATGGGCACTTTTGCGC
5	2260	CTAACTCCAGGGAAGCCTCACC
	2401	TTTGTGTGTCTTCTGCACTAAGCG
6	2486	AGCGGGACTTGAAAGGTTTAGG
	2600	TTCCCTCACGCCTGAATACC
7	2856	GAGCCTCTCGGAGACCATCC
	3000	CCAGTCATGAACCACAATCACC
8	3162	ACTGCCCTCCTCCTCATAACTTAAAGGG
	3260	CAACATCTTGGGAGAACGGTC
9	3472	TCGGAGACTAAAGAGGATTCGG
	3613	CAACATCTTGGGAGAACGGTC
10	4390	CTGTAGAAACAACAGCGGGAGTG
	4536	CTGGGCTGCTCTCAGAATCAG
11	5298	CTCAGCCATCCTCCTCGAAC
	5445	AGTGGATTGAACCCATTTCACAG

Recombinant glutathione *S*-transferase (GST)–AHSP, a gift from Mitchell Weiss, was prepared as described previously (25).

DNase I hypersensitive site mapping. DNase I HS mapping was performed as described previously (45, 70), with minor modifications, with 10^7 murine fetal liver cell nuclei. For HS mapping, DNA from embryonic stem cells was digested with BamHI for Southern blot analysis using a 211-bp fragment containing exons 1 and 2 of the AHSP cDNA as a probe. For fine mapping, the migration of the band generated by DNase I and BamHI enzyme digestion was compared with the migration of bands generated by the digestion of high-molecular-weight embryonic stem cell DNA digested with BamHI and AflIII, BlnI, HindIII, NsiI, or SmlI, respectively.

Quantitative ChIP assay. ChIP analysis of diacetylated histone H3 and tetraacetylated histone H4 was performed with antibodies from Upstate Biotechnology (06-599 and 06-866; Lake Placid, NY) as described previously (33). After elution and extraction, immunoprecipitated DNA was analyzed by quantitative real-time PCR (iCycler; Bio-Rad) as described previously using primers in Table 2 (33). Signals of test genes were normalized to a region from the murine α-globin promoter with a region from the keratin gene promoter utilized as a nonerythroid internal control. SYBR green fluorescence in 25-μl PCR mixtures was determined, and the amount of product was determined relative to a standard curve generated from a titration of input chromatin. Amplification of a single amplification product was confirmed by dissociation curve analysis and acrylamide gel electrophoresis. Samples from at least three independent immunoprecipitations were analyzed. Parallel controls for each experiment included samples of no chromatin, no antibody, preimmune serum, and nonimmune rabbit immunoglobulin G.

ChIP analysis of EKLF binding in wild-type fetal liver cells was performed with chromatin immunoprecipitated from mice with a hemagglutinin (HA)-EKLF-tandem affinity purification (TAP)-tagged knock-in allele (77) using an HA antibody (sc-7392; Santa Cruz). These mice have an HA tag knocked into the 5′ end of the EKLF gene, producing a functional EKLF protein with an HA tag at the NH_2 terminus of the protein. ChIP was performed as described previously (13), except chromatin DNA from 1 million cells was used in each reaction. Quantitative PCR amplification was performed as described above.

TABLE 3. Electrophoretic mobility shift assay probes

Probe	Sequence (5′–3′)
AHSP 3′ flanking EKLF consensus site 1	AGGATTCGGGGGGGGGGGGGAGGC GGGA TCCCGCCTCCCCCCCCCCCCCGAATCCT
AHSP 3′ flanking EKLF consensus site 2	AGCACATACCCCCACCCCCACGGCCA TGGCCGTGGGGGTGGGGGTATGTGCT
AHSP 3′ flanking EKLF consensus site 3	CCACCCCACCCCACCCCCACAGAAT ATTCTGTGGGGGTGGGGTGGGGTGG
AHSP 3′ flanking EKLF consensus site 4	TGCTTGCCCACACCCGCCAGTTTC GAAACTGGCGGGTGTGGGCAAGCA
AHSP promoter CACCC box	ACCTTCTCCACCCTAGGATG CATCCTAGGGTGGAGAAGGT
β-Globin promoter CACCC box (wild type)	TAGAGCCACACCCTGGTAAG CTTACCAGGGTGTGGCTCTA
β-Globin promoter CACCC box (C to T)	TAGAGCTACACCCTGGTAAG CTTACCAGGGTGTAGCTCTA
β-Globin promoter CACCC box (C to A)	TAGAGCAACACCCTGGTAAG CTTACCAGGGTGTTGCTCTA
β-Globin promoter CACCC box (C to G)	TAGAGCGACACCCTGGTAAG CTTACCAGGGTGTCGCTCTA
γ-Globin promoter CACCC box	TGGCTAAACTCCACCCATGGGTTG CCAGAAGCGAGTGTGTGGAACTGCT

EMSA. Recombinant GST-EKLF fusion protein was prepared using a GST-EKLF plasmid (clone C10) as described previously (48). Binding reactions, electrophoresis, and autoradiography were carried out as described previously (70). Oligonucleotide probes are shown in Table 3. Unlabeled competitor oligonucleotides were added at various molar excesses as described previously (22). EKLF antibody 6B3 was obtained from James Bieker. Quantitative electrophoretic mobility shift analyses (EMSA) was performed as described (27, 34, 73). Specifically, the fraction of free DNA, D/D_t, was determined by measuring the ratio of free DNA signal analyzed at each protein concentration at the DNA signal in a control lane containing no protein. The fraction of DNA in complex with protein, PD/D_t, was derived from the relationship $PD/D_t = 1 - D/D_t$. To derive the equilibrium dissociation constant (K_D) with standard error, the data were fit to the rearranged mass action equation, $PD/D_t = 1/(1 + K_D P)$, using nonlinear least-square analyses. Multiple analyses (three or more) were performed with the same range of protein concentrations to provide mean and standard error values for each point.

Transactivation analyses. HS2-β-globin gene promoter-luc and HS2-γ-globin gene promoter-luc plasmids were used as positive and negative controls, respectively, in transactivation assays (4, 32). The β-globin gene promoter was removed from the HS2-β-globin-luc plasmid and replaced with either a promoter frag-

TABLE 4. Expression of differentially expressed nonglobin erythroid genes in EKLF-deficient fetal liver mRNA

Genotype	Quantitative RT-PCR[a]		RNase protection ($EKLF^{-/-}$)[a]
	$EKLF^{+/-}$	$EKLF^{-/-}$	
AHSP	86.0 ± 2.8	0.0001 ± 0.0	5.0 ± 2.6
Protein 4.9	65.0 ± 1.4	1.0 ± 0.0	8.9 ± 4.3
Rh30	86.0 ± 7.1	61.5 ± 4.9	77.4 ± 9.6
Aquaporin	45.0 ± 1.4	46.0 ± 2.8	54.1 ± 8.9
Protein 4.2	86.5 ± 24.7	66.0 ± 4.2	87.6 ± 13.6
P55	66.0 ± 1.4	76.5 ± 2.1	88.3 ± 12.3
ERMAP	112.5 ± 14.8	62.0 ± 4.2	91.0 ± 17.4
Actin	97.5 ± 7.8	116.5 ± 3.5	

[a] Results are corrected with α-globin. Numbers shown are percentages of wild-type expression.

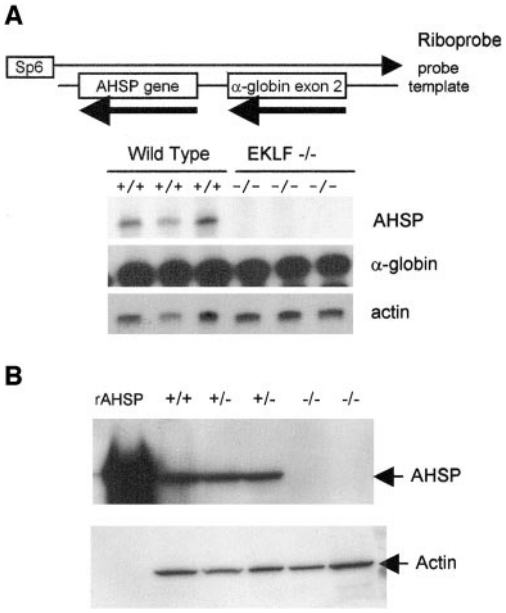

FIG. 1. Target gene expression in wild-type and EKLF-deficient fetal livers. (A) RPA of wild-type and EKLF-deficient fetal liver RNA demonstrated a 95% ± 6.2% decrease in AHSP mRNA in EKLF-deficient cells. (B) Western blot analysis of fetal liver proteins with an anti-AHSP monoclonal antibody. Recombinant AHSP (rAHSP) was added as a positive control. Virtually no AHSP was found in EKLF-deficient cells.

ment −170/+269 from the human AHSP gene (21) or a β-globin promoter with a thalassemia-associated CACCC box mutation known to perturb EKLF binding (22). Integrity of all test plasmids was confirmed by sequencing. Transient K562 cell (ATCC, CCL 243) transfections were performed as described previously with 20 μg of test plasmid, 10 μg of an EKLF expression plasmid, (48), and 0.5 μg of pCMVβ, a mammalian reporter plasmid expressing β-galactosidase driven by the human cytomegalovirus immediate-early gene promoter (Clontech) as described previously (70, 74). At least two preparations of each plasmid were tested in triplicate.

RESULTS

β-Globin and alpha-hemoglobin stabilizing protein expression are decreased in EKLF-deficient fetal liver. To identify potential EKLF target genes, subtractive hybridization was performed with total RNA isolated from day 13.5 fetal livers of wild-type (WT) and EKLF-deficient mice (78). This method has successfully been applied to a large number of applications, including gene expression in cancer, development, and hematopoiesis (28, 30, 62). In the subtraction utilizing wild-type RNA as the tester population and EKLF-deficient fetal liver RNA as the driver, ~175 differentially expressed clones were identified and subjected to sequence analysis. Validating the subtractive approach, the most abundant clone isolated corre-

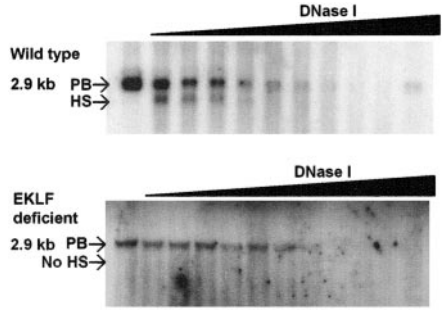

FIG. 2. Hypersensitive site mapping across the AHSP locus. (Upper panel) In chromatin from E13.5 wild-type fetal liver nuclei digested with DNase I and BamHI (B), a strong DNase I HS was found in the 5′ flanking DNA of the AHSP gene generated from a 2.9-kb parent band (PB). (Lower panel) This HS site was absent in chromatin from EKLF-deficient fetal liver.

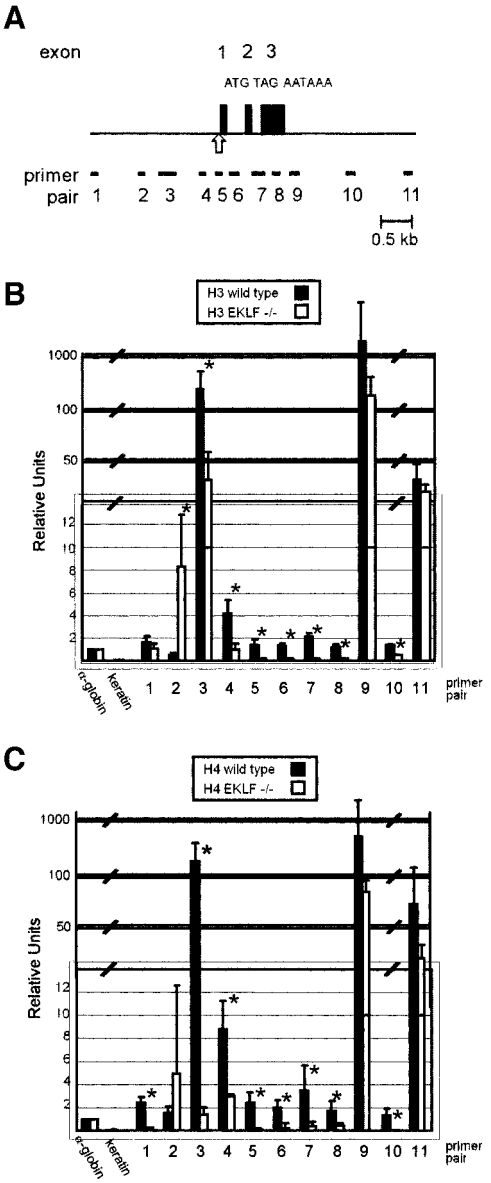

FIG. 3. Histone acetylation across the murine AHSP locus in vivo. (A) Locations of primers used for quantitative PCR amplification after chromatin immunoprecipitation of wild-type and EKLF-deficient fetal liver chromatin. The DNase I hypersensitive site in the core promoter region is denoted by the arrow. (B) Pattern of acetylation of diacetylated histone H3. (C) Pattern of acetylation of tetraacetylated histone H4. In panels B and C, differences between wild-type and EKLF-deficient chromatin with P values of <0.15 are denoted by asterisks and the values are provided in Table 5.

TABLE 5. Histone acetylation across the murine AHSP locus: dependence on EKLF

Primer[a]	H4			H3		
	WT[b]	EKLF$^{-/-b}$	*P* value	WT[b]	EKLF$^{-/-b}$	*P* value
α-Globin	1.0	1.0		1.0	1.0	
Keratin 14	0.065 ± 0.067	0.023 ± 0.01	0.34	0.05 ± 0.01	0.007 ± 0.0005	0.003
1	2.39 ± 0.53	0.25 ± 0.001	0.01	1.68 ± 0.48	1.07 ± 0.43	0.22
2	1.52 ± 0.61	4.91 ± 7.7	0.45	0.52 ± 0.18	8.27 ± 4.61	0.08
3	187.3 ± 54.5	1.41 ± 0.66	0.03	297.6 ± 9.6	38.3 ± 37.41	0.003
4	8.77 ± 2.5	2.97 ± 0.15	0.02	4.17 ± 1.19	0.99 ± 0.56	0.004
5	2.36 ± 0.94	0.11 ± 0.12	0.047	1.39 ± 0.47	0.077 ± 0.10	0.03
6	1.96 ± 0.67	0.23 ± 0.42	0.01	1.33 ± 0.21	0.103 ± 0.11	0.003
7	3.46 ± 2.18	0.43 ± 0.34	0.13	2.1 ± 0.38	0.09 ± 0.08	0.01
8	1.73 ± 0.83	0.49 ± 0.20	0.11	1.22 ± 0.26	0.17 ± 0.04	0.01
9	819.7 ± 250.6	78.4 ± 11.9	0.04	1,099.9 ± 444.9	144.2 ± 75.6	0.45
10	1.31 ± 0.59	0.06 ± 0.005	0.07	1.37 ± 0.11	0.55 ± 0.01	0.008
11	70.6 ± 42.3	19.0 ± 15.6	0.24	37.8 ± 15.6	35.13 ± 7.84	0.86

[a] Primer pairs correspond to Table 2 and Fig. 3A.
[b] Numbers are expressed as relative units, normalized to α-globin as 1.

sponded to β-globin ($n = 122$). The second most abundant clone isolated was AHSP ($n = 22$). No other single clone except G protein Gi2a ($n = 8$) was represented by more than 5 clones. Results of subtraction were compared to results of differential gene expression in EKLF-deficient cells identified by analysis of a noncommercial microarray (20). Both techniques identified β-globin, AHSP, and the membrane protein gene protein 4.9 (3 clones in the subtraction). Compared to the array, subtraction did not identify hemogen, hemoglobin Z, any enzymes involved in heme biosynthesis, and several membrane-associated genes, such as those for Kell, CD24a, Icam4, and Kcnn4. It did identify other membrane-associated proteins, such as aquaporin, Rh30, p55, protein 4.2, and ERMAP.

When subtraction was performed using EKLF-deficient RNA as the tester population and wild-type fetal liver RNA as the driver, only 12 clones were obtained. No clone was represented more than once, even though EKLF has been shown to have a repressor function (14, 15).

Reduced expression of potential target genes in EKLF-deficient fetal liver RNA was confirmed using either quantitative, real-time RT-PCR (Table 4) or RPA. The most frequently isolated clone after β-globin, AHSP, had nearly undetectable mRNA levels in EKLF-deficient RNA (Table 4; Fig. 1A). Western blot analysis with a rat anti-mouse monoclonal antibody against AHSP demonstrated that there was no AHSP in day 13.5 fetal livers from EKLF-deficient mice (Fig. 1B). Other genes with significantly decreased mRNA levels in EKLF-deficient mRNA included protein 4.9, which was also nearly undetectable, and aquaporin, reduced to less than 50% of the wild-type level (Table 4). Because AHSP was the most significantly altered gene in the differential subtraction, we selected it for further study.

Chromatin at the AHSP locus is altered in EKLF-deficient mice. EKLF interacts with the proximal CACCC box of the β-globin gene promoter, establishing local chromatin structure and directing high-level β-globin transcription. In EKLF-deficient cells, there is loss of the DNase I hypersensitive site at the β-globin promoter and diminution of another HS site (HS3) over 50 kb away in the β-globin locus control region (26, 69). Thus, we hypothesized that chromatin across the AHSP locus would be perturbed in erythroid cells from EKLF-deficient mice. To interrogate the chromatin status of the AHSP gene, DNase I hypersensitive site mapping and ChIP were performed.

In chromatin from E13.5 WT fetal liver nuclei, a strong DNase I HS was demonstrated in the immediate 5′ flanking DNA in the core promoter region in a 162-bp HindIII/SmlI fragment (Fig. 2, upper panel) corresponding to coordinates 2187 to 2349 of a 6,472-bp murine AHSP fragment (GenBank accession no. AF485327) (59). Fine mapping of the HS localized it to a 43-bp HindIII/SmaI fragment corresponding to the core AHSP promoter containing a CACCC box (not shown). This HS site was absent in chromatin from EKLF-deficient fetal livers (Fig. 2, lower panel).

Histone modifications across 3.5 kb of the murine AHSP locus were examined using a ChIP assay with anti-diacetyl histone H3 and anti-tetraacetyl histone H4 antibodies and fetal liver chromatin from E13.5 WT and EKLF-deficient embryos. Eleven primer pairs spanning the murine AHSP locus, approximately 300 bp apart (Table 2 and Fig. 3A), were utilized for PCR. ChIP analysis of WT fetal liver chromatin demonstrated two regions where histones H3 and H4 were hyperacetylated relative to a control region from the mouse α-globin gene promoter (Fig. 3B and C; Table 5). The 5′ region corresponded to the 5′ flanking DNA and promoter region of the AHSP gene, and the second region mapped 3′ of the region of the AHSP cDNA in proximity to the polyadenylation signal (Fig. 3). Compared to the control, histones H3 and H4 were also acetylated in the interval between these peaks of hyperacetylation. In chromatin immunoprecipitated from EKLF-deficient fetal liver cells, there was hypoacetylation between primer pairs 4 through 8 (Fig. 3B and C; Table 5). This encompasses the core AHSP promoter and coding region, correlating with the severe reduction in AHSP gene expression. Significant H3 acetylation extends 5′ of the AHSP core promoter region, potentially indicating the interaction of EKLF with other regulatory elements in this region.

EKLF binds to a site in the 3′ region of the AHSP gene in vitro. Analysis of the murine AHSP gene including 10 kb 5′ and 3′ of the coding region identified four canonical EKLF binding consensus sites, CCNCNCCCN, in the 3′ flanking region. To determine if EKLF could bind any of these EKLF

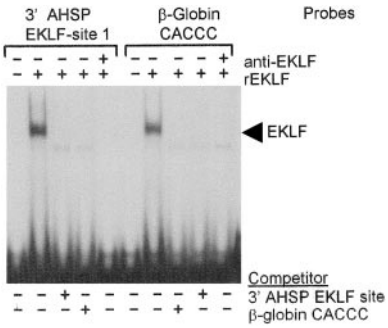

FIG. 4. Gel mobility shift assays of the EKLF consensus binding sites in the 3′ region of the AHSP gene. Gel mobility shift assays using oligonucleotide probes corresponding to the EKLF consensus binding sites in the 3′ flanking region of the murine AHSP gene were performed using recombinant EKLF protein (rEKLF). Results with site 1 are shown. A β-globin promoter-proximal CACCC box probe was used as a positive control. Excess, unlabeled probe or EKLF antibody was added where indicated. +, present; −, absent.

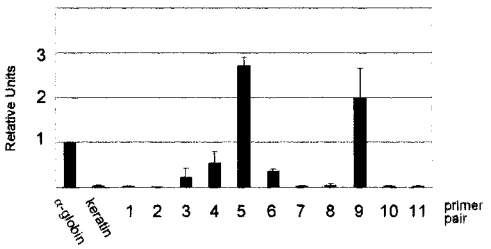

FIG. 5. EKLF occupancy across the murine AHSP locus in vivo. Quantitative chromatin immunoprecipitation across the AHSP locus was performed with fetal liver chromatin from mice with an HA-EKLF-TAP-tagged knock-in allele. Quantitative PCR amplification was performed with the primers shown in Fig. 3A.

sites in vitro, double-stranded oligonucleotide probes containing the AHSP 3′ flanking DNA EKLF sites or a β-globin promoter EKLF binding site probe as a positive control were prepared and used in EMSA with recombinant EKLF protein. The AHSP probe near the AHSP polyadenylation signal (AHSP 3′ flanking EKLF consensus site 1) (Table 3) yielded a single complex that migrated identically to a complex formed by the control β-globin promoter probe (Fig. 4). These complexes were effectively competed by both an excess of unlabeled AHSP probe, an excess of unlabeled control β-globin probe, and a monoclonal antibody against EKLF protein (not shown). The other three probes did not bind to recombinant EKLF (not shown). These data indicate that EKLF binds to a region of the 3′ flanking DNA region of the AHSP gene in vitro. This site is in the region of histone hyperacetylation identified by ChIP (primer pair 9) (Tables 2 and 5; Fig. 3).

EKLF binds to the AHSP promoter CACCC box in vivo. We wished to determine the region(s) where EKLF bound to the AHSP gene in vivo. However, we were unable to establish conditions for ChIP with anti-EKLF antibodies. Instead, ChIP across the AHSP locus was performed with HA-immunoprecipitated chromatin obtained from mice with an HA-EKLF-TAP-tagged knock-in allele (77). A peak of binding was seen in the 3′ region of the AHSP gene where a region of histone hyperacetylation was identified by ChIP and binding to a canonical EKLF consensus site was found (Fig. 5; Table 6).

Another region of EKLF binding extending from the 5′ region of the AHSP promoter HS to intron 1, peaking over the site of the AHSP promoter CACCC box, was identified (Fig. 5; Table 6). This peak is over the region of the DNase I hypersensitive site and histone hyperacetylation found in wild-type fetal liver cells.

A CACCC box in the AHSP gene promoter binds EKLF in vitro. The AHSP promoter CACCC box, located in the region of the DNase I hypersensitive site, histone hyperacetylation, and EKLF binding, did not match the EKLF binding consensus, CCNCNCCCN, as it had a single mismatch at position 1, ACCCACCCT. To determine if EKLF could bind the imperfect EKLF site in the AHSP gene promoter in vitro, double-stranded oligonucleotide probes containing the AHSP 5′ flanking DNA imperfect EKLF site or a β-globin promoter EKLF binding site probe were prepared and used in EMSA with recombinant EKLF protein. The AHSP probe yielded a single complex that migrated identically to a complex formed by the control β-globin promoter probe (Fig. 6A). These complexes were effectively competed both by an excess of unlabeled AHSP probe, an excess of unlabeled control β-globin probe, and a monoclonal antibody against EKLF protein (Fig. 6A). These data indicate that EKLF binds to a region of the 5′ flanking DNA region of the AHSP gene in vitro.

To examine the influence of position 1 on EKLF-DNA binding, a β-globin oligonucleotide probe with a mutation of the EKLF consensus sequence to mimic the AHSP sequence (position 1, C to A) (Fig. 6B) or probes with mutation of position 1 to the other two possibilities (C to G and C to T) (Fig. 6C) were used in EMSA. All test probes yielded complexes similar to that of the wild-type β-globin probe. These complexes were effectively competed by an excess of unlabeled AHSP probe, an excess of unlabeled control β-globin probe, and a monoclonal antibody against EKLF protein (Fig. 6B and C). These data indicate that the C at position 1 of the EKLF consensus site is not critical for EKLF binding to the β-globin proximal CACCC box in vitro.

Competitive electrophoretic mobility shift assays were performed to compare the β-globin promoter CACCC site to the

TABLE 6. EKLF occupancy across the murine AHSP locus determined by ChIP

Primer[a]	HA-EKLF	*P* value
α-Globin	1.0	
Keratin 14	0.034 ± 0.016	
1[b]	0.018 ± 0.017	
2	0.01 ± 0.000	0.39
3	0.22 ± 0.21	0.25
4	0.55 ± 0.24	0.04
5	2.7 ± 2.0	0.15
6	0.36 ± 0.04	0.001
7	0.03 ± 0.01	0.31
8	0.04 ± 0.04	0.49
9	2.0 ± 0.65	0.03
10	0.03 ± 0.01	0.54
11	0.02 ± 0.01	0.90

[a] Primer pairs correspond to Table 2 and Fig. 3A.
[b] Internal standard.

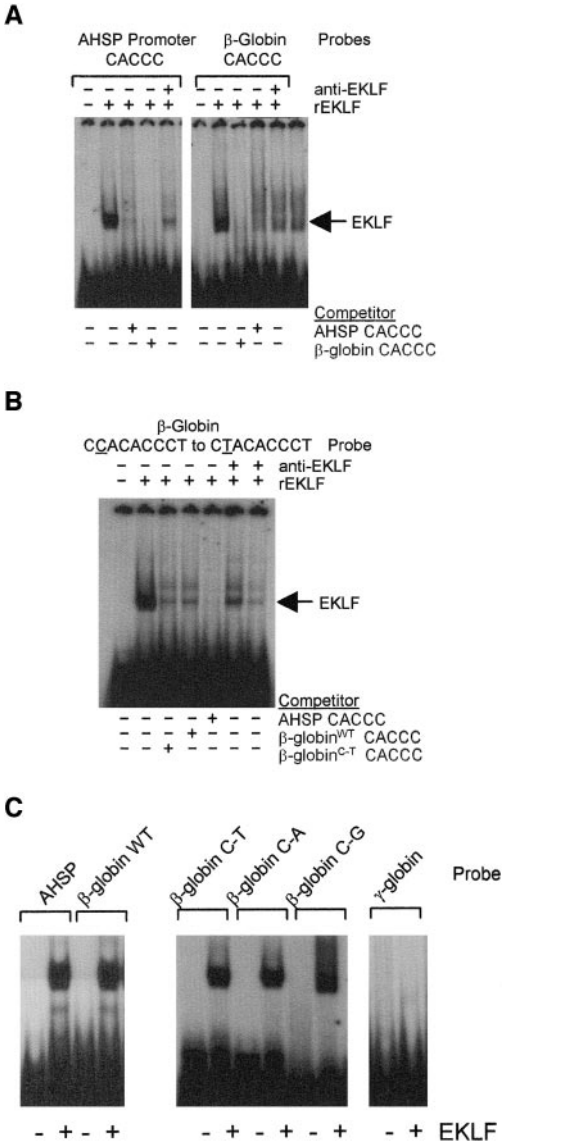

FIG. 6. Gel mobility shift assays of the AHSP promoter CACCC box. Gel mobility shift assays using oligonucleotide probes were performed using recombinant EKLF protein (rEKLF). Excess, unlabeled probe or EKLF antibody was added where indicated. (A) Probes corresponding to the AHSP promoter CACCC box and the β-globin promoter-proximal CACCC box. (B) A β-globin CACCC box probe with position 1 mutated from C to T to mimic the AHSP CACCC box. (C) Probes corresponding to the AHSP promoter CACCC box, the wild-type β-globin CACCC box, the β-globin promoter CACCC box with position 1 of the EKLF consensus sequence mutated to the other 3 possible nucleotides, and the γ-globin promoter CACCC box. +, present; −, absent.

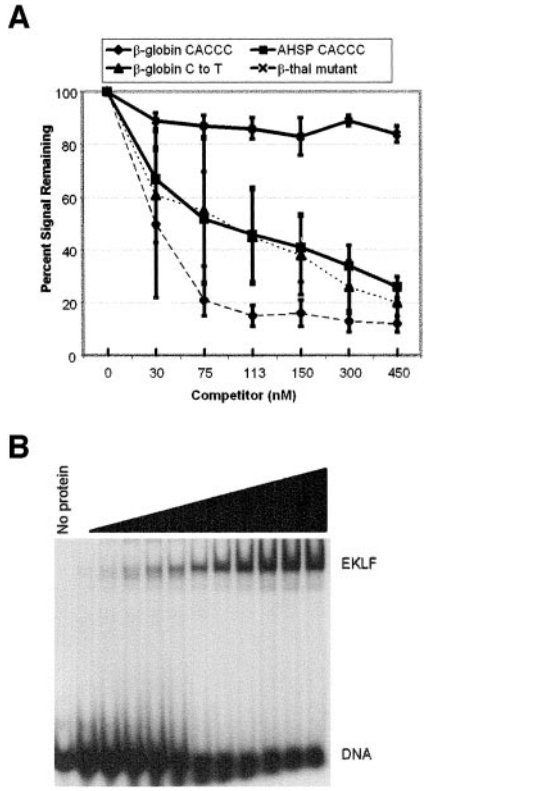

FIG. 7. Quantitative electrophoretic mobility shift assays of the AHSP promoter CACCC box. (A) Competitive electrophoretic mobility shift assays were performed with the β-globin promoter CACCC box as a probe. Different unlabeled probes are assayed for their ability to compete the EKLF-CACCC complex. The amount of complex without competitor is defined as 100%. The points where curves cross the 50% line of the percent signal remaining was used as estimate the competitive ability of each oligonucleotide probe for binding to EKLF relative to the wild-type β-globin promoter CACCC box. β-thal, β-thalassemia. (B) EKLF protein titrations with β-globin and AHSP CACCC boxes. To determine the K_D for the interaction between EKLF and each CACCC box, EKLF protein titrations were performed, gels scanned, and K_D calculated with the rearranged mass action equation, $PD/D_t = 1/(1 + K_D P)$, using nonlinear least-square analyses. A sample gel with a wild-type β-globin promoter CACCC box probe is shown.

AHSP CACCC site. In this assay, the wild-type β-globin promoter CACCC site is used as a probe and different unlabeled probes are assayed for their ability to compete the EKLF-CACCC complex. Approximately 50 nM unlabeled wild-type β-globin CACCC probe was required to compete the EKLF-CACCC complex by 50% (Fig. 7A). Approximately 150 nM unlabeled wild-type AHSP probe or β-globin CACCC probe with position 2 C to T was required for similar competition (Fig. 7A). An unlabeled β-globin CACCC probe with a thalassemia mutation known to perturb EKLF binding (CACCC to CACGC) did not compete the EKLF-CACCC complex at the amounts added in these experiments. Quantitative EMSA were performed to further assess binding of the AHSP CACCC site. Recombinant EKLF-GST expressed in *E. coli* and purified on glutathione-Sepharose beads was used in DNA titration experiments to determine the concentration of protein capable of binding DNA (not shown). Active protein concentrations were then used in protein titrations to determine the K_D for the various CACCC-containing oligonucleotide probes of interest (Fig. 7B). The highest-affinity site was the β-globin promoter CACCC box, with a K_D of $3.98 \times 10^{-4} \pm 0.91 \times 10^{-4}$ nM. The AHSP promoter CACCC box and the β-globin promoter CACCC boxes with position 2 mutated had affinities 30- to 40-fold less than the β-globin promoter CACCC box: AHSP promoter CACCC box, $1.1 \times 10^{-3} \pm 0.90 \times 10^{-3}$ nM; β-globin

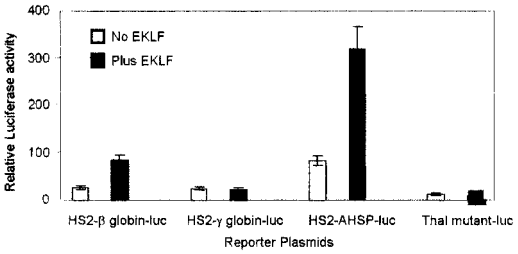

FIG. 8. EKLF transactivates the AHSP promoter in K562 cells. HS2-AHSP promoter, HS2-γ-globin promoter, HS2-β-globin gene promoter, or mutant HS2-β-globin gene promoter/luciferase reporter plasmids were cotransfected into K562 cells with an EKLF cDNA expression plasmid. Luciferase activity was assayed 24 h after transfection and normalized to β-galactosidase to control for transfection efficiency. Thal, thalassemia.

C to T, $1.1 \times 10^{-3} \pm 0.18 \times 10^{-3}$ nM; β-globin C to A, $1.3 \times 10^{-3} \pm 0.46 \times 10^{-3}$ nM; and β-globin C to G, $1.2 \times 10^{-3} \pm 0.36 \times 10^{-3}$ nM. The β-globin thalassemia mutant demonstrated a significantly lower binding affinity than the wild-type β-globin promoter CACCC box, $4.6 \times 10^{-2} + 0.39 \times 10^{-2}$. Together, these data indicate that the C at position 2 of the EKLF consensus site influences, but is not absolutely essential for, EKLF binding to the β-globin proximal CACCC box in vitro.

Transactivation of the AHSP gene promoter by EKLF in K562 cells. Luciferase reporter plasmids with an HS2-AHSP gene promoter fragment, (24) an HS2 human β-globin promoter fragment as a positive control, and an HS2 γ-globin promoter fragment as a negative control (4, 32) were transiently cotransfected into K562 cells with an EKLF cDNA expression plasmid. The AHSP promoter and the positive control β-globin promoter plasmids were transactivated by EKLF, whereas the negative control γ-globin promoter and thalassemia-mutant β-globin promoter plasmids were not (Fig. 8).

DISCUSSION

Identification of AHSP as an EKLF target gene confirms the finding of differential expression identified by analysis of a noncommercial microarray (20) in a different genetically altered mouse line (57) and suggests that the hemolytic anemia in EKLF-deficient mice is due to both decreased transcription of the β-globin gene and decreased expression of other erythroid genes. AHSP deficiency could be part of the explanation why simply balancing globin chain synthesis does not rescue EKLF-deficient mice. Interestingly, in several studies of β-thalassemia patients with varying clinical severity, no mutations were identified in the AHSP gene (12, 23, 41, 68), even though in some reports discordant thalassemic patients were found to have decreased AHSP mRNA or protein expression in erythroid cells (23, 41). It is possible that the variation in clinical severity and AHSP expression are attributable to differences in EKLF, making EKLF another candidate modifier gene in the β-thalassemia syndromes. Deficiency of other erythroid genes such as protein 4.9 or other membrane proteins may also contribute to this anemia.

Like the β-globin locus, the AHSP locus demonstrates significant alterations in chromatin configuration in EKLF-deficient cells. Alterations in chromatin were not only found in the core AHSP promoter region at a site of EKLF-DNA binding but across the locus where there was global histone hypoacetylation. EKLF plays an important role in chromatin remodeling at the β-globin locus (1, 26, 36, 42, 47, 69, 75). EKLF is acetylated by CBP and p300, coactivator proteins that posses histone acetyltransferase activity (9, 75). It also interacts with other proteins, including those of the SWI/SNF chromatin remodeling complex (1, 10, 36, 42, 76). Recent evidence demonstrates that EKLF plays an important role in formation of an active chromatin hub in erythroid cells (21). Together, these data demonstrate support for the hypothesis that chromatin remodeling of the AHSP locus requires EKLF and demonstrate that EKLF may act as a transcription factor and a chromatin modulator for genes other than β-globin.

These data also demonstrate the value of an HA-EKLF-TAP-tagged knock-in allele mouse. In vivo histone acetylation data demonstrated two regions of hyperacetylation in wild-type mice that were absent in EKLF-deficient mice, one over the promoter and the other in the 3′ region of the gene. In vitro EMSA studies suggested that EKLF binding was occurring in the 3′ region. Generation of in vivo data from the HA-EKLF-TAP-tagged mouse permitted identification of EKLF binding to the core promoter CACCC box, which based on the published EKLF consensus sequence, was not initially considered.

The EKLF consensus sequence CCNCNCCCN was generated from data obtained from modeling of the crystal structure of DNA zinc finger contacts of Zif268 at 2.1 Å, as well as in vivo footprinting and methylation interference studies of the murine β-globin promoter (22, 54, 60). These studies demonstrated that β-thalassemia mutations associated with the β-globin promoter CACCC box disrupt specific contacts between guanine on the G-rich strand and arginine or histidine of the XYZ of fingers 2 or 3 (22). Identification of an EKLF binding site with a substitution in finger 1, where no thalassemia-associated mutations have been identified, suggests some relaxed freedom for DNA binding of finger 1 by EKLF. Finger 1 of EKLF is similar to another member of the C_2H_2-type zinc finger protein family, the ubiquitous transcription factor Sp1. Compared to fingers 2 and 3, finger 1 of Sp1 has more relaxed sequence and site specificity and contributes less to its DNA binding affinity (51, 67, 72).

Recent nuclear magnetic resonance structure of Sp1 revealed that, compared to fingers 2 and 3, which recognize four DNA base pairs by residues −1, 2, 3, and 6 of the recognition helix, finger 1 uses only residues −1 and 3 for DNA recognition (51). Differences in 3 amino acids of Sp1 finger 1 contribute to a broader recognition sequence than Zif268 and other C_2H_2-type zinc finger proteins. EKLF has a sequence identical to that of Sp1 at these 3 residues (see Fig. S1 in the supplemental material). Based on these data, it is possible that the EKLF consensus is more similar to the GGGCCG consensus of Sp1, e.g., NCNCCC, and that other factors, such as sequence context, composition of basal transcription machinery recruited to the promoter, and/or other EKLF-protein interactions, determine specificity and activity (4, 8). Several reports support this hypothesis, demonstrating that the function of the β-globin CACCC box is context dependent (4, 18, 29, 43, 64).

ACKNOWLEDGMENTS

This work was supported in part by grants HL68965 (T.M.T.), HL65448, and DK/HL62039 (P.G.G.) from the National Institutes of Health and intramural funds from NHGRI.

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