α-Hemoglobin-stabilizing protein (AHSP, also known as erythroid differentiation-related factor, EDRF, and erythroid-associated factor, ERAF) is a recently identified erythroid protein that binds free α-hemoglobin and prevents its precipitation in erythroid precursors, damaging the membrane and triggering cell death (5). Thus AHSP is critical during normal erythropoiesis, where α-globin chain synthesis normally exceeds β-globin chain synthesis, and to a greater extent during pathologic states of globin chain imbalance. Because of its role in stabilizing α-globin, it has been suggested that AHSP may be a modifier gene in the β-thalassemia syndromes (1, 6). Decreased or defective AHSP would be expected to worsen the phenotype of β-thalassemia because of the cytotoxic effects of excessive free α-hemoglobin. In a murine model, AHSP deficiency leads to well-compensated hemolysis with Heinz body formation, reticulocytosis, and increased apoptosis of erythroid precursors (5). When AHSP-deficient mice were bred to β-thalassemia mice, loss of AHSP increased the severity of the thalassemia (5). These studies also suggested that AHSP might play a role in unexplained Heinz body hemolytic anemia in humans.

Identification and characterization of the regulatory elements that control AHSP gene expression have important implications for normal erythropoiesis and the pathogenesis of hemolytic disorders. AHSP may have therapeutic potential for β-thalassemia patients by decreasing α-globin precipitation and ameliorating clinical severity. Because AHSP is synthesized abundantly in erythroid cells, identification of the regulatory elements directing this high level, tissue-specific expression may provide important tools in directing other erythroid-specific genes in gene transfer applications.

AHSP is a highly expressed and erythroid-specific protein (1, 7, 8). It is present throughout primitive and definitive erythropoiesis of the mouse and accumulates to high levels in late erythroid cells. Its pattern of expression follows that of the α-globin gene throughout erythropoiesis (8). Identified as a target of the critical erythroid transcription factor GATA-1 (1), the AHSP gene is highly and rapidly induced by GATA-1.

In this report, we describe the identification and characterization of the human AHSP gene promoter. Our results demonstrate that the AHSP gene promoter requires GATA-1- and Oct-1-binding proteins to direct high level expression in erythroid cells in vitro. The minimal promoter, −170 to +269, includes the 5′-flanking DNA and intron 1. In transgenic mice, a reporter gene directed by the AHSP minimal promoter directed expression exclusively in erythroid cells. The results demonstrate that the region from −170 to +269 contains the sequences necessary for expression in erythroid cells and that this expression is dependent on GATA-1.

**MATERIALS AND METHODS**

**Genomic Cloning**—A human genomic library in PACs was screened by PCR amplification with oligonucleotide primers, 5′-GAGATTCCACGCACCTCAAGAGTG-3′ (Pα sense) and 5′-GCAAGACCGTGAATCTCTTAA-3′ (Pα antisense) (Fig. 1A), as described (9). These primers correspond to the 5′-end of the AHSP cDNA and amplify a 299-bp product from human genomic DNA.

**5′-RACE (Rapid Amplification of cDNA Ends)**—Total RNA was prepared from human bone marrow or from the human tissue culture cell lines K562 (chronic myelogenous leukemia in blast crisis with erythroid characteristics, ATCC, CCL 243) or HeLa (epithelial-like carcinoma, cervix, CCL 2). 1 μg of total RNA was reverse-transcribed with an antisense primer, 5′-CTGTCGATTTCAGACGAAAGCCTGAAAC-3′ and AMV reverse transcriptase (Promega). Single-stranded oligonucleotide

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**References**

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2. The abbreviations used are: AHSP, α-hemoglobin-stabilizing protein; RACE, rapid amplification of cDNA ends; EMSA, electrophoretic mobility shift analyses; CHIP, chromatin immunoprecipitation assay.
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**FIGURE 1. The human AHSP gene.** A, structure of the 5'-end of the human AHSP cDNA. A diagram of the 5'-end of the human AHSP cDNA is shown. The 5'-end of the previously reported sequence obtained from cDNA cloning is shown. Sequences obtained by 5'-RACE are denoted by the box. The initiation and termination codons are shown. B, genomic organization of the 5'-end of the human AHSP gene. One clone containing the AHSP gene was isolated from a human genomic DNA library. This clone spanned a distance of ~130 kb and contained exons 1–3 of the AHSP cDNA in a 11-kb EcoRI (E) fragment. The location of the PCR primers used in genomic library screening, P<sub>S</sub> and P<sub>A</sub>, are shown. C, mapping the 5'-end of the human AHSP cDNA. Primer extension was carried out using 20 μg of K562 or HeLa cell total RNA or 1 μg of RNA as template. Lanes AGCT, nucleotide markers; lane 1, labeled primer plus K562 cell RNA; lane 2, labeled primer plus HeLa cell RNA; lane 3, labeled primer plus RNA. The size of the extension products (lane 1, arrow) indicates that the 5'-end of the mRNA is located 129-bp upstream relative to the 3'-end of the primer and 32-bp upstream of the previously reported 5'-end of the AHSP cDNA. The CDNA sequence of this additional 5'-untranslated cDNA was determined by 5'-RACE. D, 5'-flanking genomic DNA sequence. The nucleotide sequence of the 5'-flanking genomic DNA of the human AHSP gene is shown. Consensus sequences for potential GATA-1 binding sites and a potential Oct-1 binding site in intron 1 are underlined. The transcription initiation site, +1, is denoted by the arrow above the A. The sequences encoding introns 1 and 2 are shown in lowercase. The initiation codon is boxed.

ligation and PCR amplification were carried out as described using primers 5’-CCATCTTAATACGACTCACTATAGGGC-3’ (sense) and CTGCTGATTCCAGAAGCTCGTGAAC-3’ (antisense) and 5’-CCATCTTAATACGACTCACTATAGGGC-3’ (sense) and 5’-TCTCTTTGATCCGGCAATTGAGATC-3’ (antisense), respectively (Fig. 1). Amplification products were subcloned and sequenced.

**Mapping the AHSP Gene Transcription Initiation Site**—The transcription initiation site of the erythroid AHSP cDNA was determined using a primer extension assay as described with primer 5’-GGCGTG-TATATGTCCTACCCACACTCTTTG-3’ and 20 μg of total K562 or HeLa cell RNA or 1 μg of RNA (11).

**Transient Transfection Analyses—AHSP promoter fragments** −902/+32, −479/+32, −170/+32, −904/+269, −479/+269, and −170/+269 were amplified by PCR using the human AHSP genomic DNA clone as template. These fragments were subcloned upstream of the firefly luciferase reporter gene in the plasmid pGL2B (Promega). Integrity of all test plasmids was confirmed by sequencing. Plasmids were purified using Qiagen columns (Qiagen, Inc., Chatsworth, CA), and at least two preparations of each plasmid were tested in triplicate. K562 and Hela cell transfections were performed as described (12).

**Preparation of Nuclear Extracts**—Nuclear extracts were prepared from K562 cells by hypotonic lysis followed by high salt extraction of nuclei as described by Andrews and Faller (13). K562 and Hela cell transfections were performed as described (12).

**DNase I Footprinting in Vitro**—The probes for DNase I footprinting were produced by PCR amplification of a subcloned AHSP genomic fragment −170/+269 as template and a pair of oligonucleotide primers, 5’-GGGCTCGAGGCTCTTCTTTCCATTTG-3’ (sense) and 5’-CCGAGGCTTCTGGGTAGAGAAGGGGTTAGA-3’ (antisense). One oligonucleotide was 5’-end-labeled with [32P]ATP using polynucleotide kinase prior to use in PCR. Reaction mixes contained K562 cell nuclear extracts, 10,000 cpm of labeled probe, and 1 μg of poly(dI-dC). After digestion with DNase I, samples were electrophoresed in 6% denaturing polyacrylamide gels, the gels dried, and subjected to autoradiography.

**Electrophoretic Mobility Shift Analyses (EMSA)**—Binding reactions were carried out as described (12). Competitor oligonucleotides were added at molar excesses of 100-fold. Antibodies to GATA-1 and Oct1 were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). Reaction mixes were electrophoresed through 6% non-denaturing polyacrylamide gels in 0.5% Tris-borate-EDTA at 21 °C at 200 watts for 2 h. Gels were dried and subjected to autoradiography.

**Quantitative Chromatin Immunoprecipitation (ChIP) Assay**—Anti-diacetylated histone H3 (06-599) and anti-tetraacetylated histone H4 (06-866) antibodies were obtained from Upstate Biotechnology (Lake Placid, NY). Anti-GATA-1 antibody (N6) was obtained from Santa Cruz. After formaldehyde fixation, chromatin was fragmented by sonication 10 times for 10 s each. Extracts were precleared with protein G- Sepharose, and incubated overnight at 4 °C with antibody. After elution and extraction, immunoprecipitated DNA was analyzed by quantitative real-time PCR (iCycler, Bio-Rad) as described (14). Primers 5’-TCCTCATTTCTTTT-3’ (sense) and 5’-CAGCTAAAGATAGAAAAGCCC-3’ (antisense) amplify across 139 bp in the AHSP minimal promoter. An inactive region from the neural-specific necdin gene was also amplified as control using primers 5’-CCCAACATC-CCCAACATC-CCCAACATC-3’ (sense) and 5’-GATCCCCGGGTTCTCTGTGTTT-3’ (antisense). Samples from at least three independent immunoprecipitations were analyzed. Amplification of a single amplification product was confirmed by dissociation curve analysis and agarose gel electrophoresis with ethidium bromide staining. Parallel controls for each experiment included samples of no chromatin, no antibody, non-immune rabbit IgG, and rabbit nonimmune serum.

**Preparation of Promoter Reporter Plasmids for Transgenic Mice**—A 439-bp AHSP gene promoter fragment from −170/+269 was ligated to a 2266-bp EcoRV/AatII fragment containing the human β-globin
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The human AHSP gene was excised from this plasmid with KpnI and HindIII for transgene copy number was determined by comparing the activity obtained from the transfectants versus the activity obtained from the promoterless plasmid pGL2B plasmid taking into account the transfection efficiency. The data are means ± S.D. of at least six independent transfection experiments. The functionally important GATA-1 site at −98 to −104 is marked by a star, B, mutations in consensus DNA-protein binding sites, marked with an X, were introduced into the AHSP promoter/luciferase reporter plasmids and transfected into K562 cells and activity determined as described.

Generation and Analyses of Transgenic Mice—Transgenic mice were generated as described (15, 16). Transgene copy number was determined by comparing the γ-globin signals from Southern blot analysis of transgenic mouse and K562 DNA using a Molecular Dynamics PhosphorImager. Statistical analysis of copy number and expression data were analyzed by linear regression using GraphPad Prism software.

Ribonuclease Protection Analysis of Transgene Expression—Expression of the human promoter/γ-globin reporter transgene was analyzed using an RNase protection assay (RPA). Probe synthesis, hybridization, electrophoresis, autoradiography, and analyses were performed as described (12).

Expression of Human γ-Globin Protein in the Erythrocytes of Transgenic Mice—Detection and measurement of γ-globin protein in red blood cells was performed as described (15).

RESULTS

Cloning of Chromosomal Gene Isolation and Analysis of Recombinant Clones—The PCR-based screening of the human genomic BAC library yielded one PCR-positive clone ~130 kb in length. DNA fragments that amplified with the screening primer pair were purified and subcloned into plasmid vectors. Restriction enzyme analysis, Southern blotting, and limited nucleotide sequencing identified the DNA fragments as AHSP gene-specific. Comparison of cDNA and genomic DNA sequences allowed determination of the genomic structure of the AHSP gene, a three exon gene spread over 130 kb in length. DNA fragments that yielded one PCR-positive clone were obtained by 5′- and the 3′-end of the third exon contain untranslated sequence. The entire first exon and the 3′-end of the third exon contain untranslated sequence. The aggt rule is not violated at any splice junction.

Mapping the Human AHSP Erythroid mRNA Transcription Initiation Site and Identiﬁcation of 5′-cDNA Sequences—To identify the 5′-end of the human AHSP cDNA, primer extension was performed using total RNA from K562 and HeLa cells. These experiments identiﬁed a single transcription initiation site in erythroid cells (Fig. 1B) and predicted the presence of an additional 32 bp in the mRNA upstream of the 5′-end of the previously reported sequence obtained from cDNA cloning. These additional 32 bp of upstream 5′-untranslated sequence were obtained by 5′-RACE. Sequences obtained by 5′-RACE were veriﬁed by comparison to corresponding genomic DNA sequences (Fig. 1C). The sequences around the transcription start site, ACACTTTG closely match transcription initiation recognition sequences, YYAACWY (17). The first ATG is in exon 2, no ATGs are present in

FIGURE 2. Activity of the AHSP gene promoter in erythroid and nonerythroid cell lines in transient transfection assays. A, plasmids containing 5′-flanking DNA of the AHSP gene inserted upstream of the firefly luciferase gene were transfected into K562 or HeLa cells as described. Relative luciferase activity was expressed as that obtained from the test plasmids versus the activity obtained from the promoterless plasmid pGL2B plasmid taking into account the transfection efficiency. The data are means ± S.D. of at least six independent transfection experiments. The functionally important GATA-1 site at −98 to −104 is marked by a star, B, mutations in consensus DNA-protein binding sites, marked with an X, were introduced into the AHSP promoter/luciferase reporter plasmids and transfected into K562 cells and activity determined as described.

FIGURE 3. A, in vitro DNase I footprinting of the human AHSP promoter. In vitro DNase I footprinting of the human AHSP gene promoter was performed using K562 extracts as described in the text. Two protected sites were identiﬁed, one in the 5′-flanking genomic DNA corresponding to a GATA-1 consensus binding site, and one in intron 1 corresponding to an Oct-1 consensus binding sequence. B, electrophoretic mobility shift assays of the GATA-1 site of the human AHSP gene promoter. Gel mobility shift assays using a probe corresponding to the GATA-1 consensus binding site of the human AHSP promoter identiﬁed in in vitro footprinting were performed using K562 nuclear extracts. Wild-type and mutant AHSP GATA-1 probes and a control GATA-1 probe were used. A GATA-1 antibody was added to the reaction mixtures where indicated.
the 5′-untranslated sequences. Taken together, these data suggest that this sequence is at or very near the 5′-end of the human AHSP erythroid cDNA.

The nucleotide sequence of the 5′-flanking genomic DNA upstream of the human AHSP cDNA transcription start site is shown in Fig. 3. Consensus TATA or CCAAT sequences are absent. Characteristic of an erythroid gene promoter, multiple consensus binding sites for GATA-1 and a single Sp1/CACCC site are present.

An AHSP Gene Promoter Fragment Is Active in Erythroid Cells—Plasmids containing 5′-flanking putative AHSP promoter sequences linked to a luciferase reporter gene were transiently transfected into K562 cells. Relative luciferase activity was determined 48 h after transfection and compared with the activity obtained with pGL2B, a negative control, the promoterless plasmid, and pGL2P, a positive control, the luciferase promoter reporter plasmid (Fig. 2 A).

Promoter fragments containing the same sequence encoding the 5′-end of the human AHSP erythroid promoter or control GATA-1 sequences (TABLE ONE) (18) were prepared and used EMSA with K562 (erythroid) extracts. The AHSP GATA-1 probe yielded a single complex that migrated identically to a complex formed with a control GATA-1 probe. These complexes were effectively competed both by an excess of unlabeled AHSP GATA-1 oligonucleotide, an excess of unlabeled control GATA-1 oligonucleotide, and a monoclonal antibody against GATA-1 protein (Fig. 3 B).

Similar to the human a-spectrin gene, GATA-1, and the erythropoietin receptor, where the addition of intron 1 sequences markedly increase promoter strength (14), we hypothesized that the addition of intron 1 would increase AHSP promoter expression. 234 bp of contiguous sequence encoding the 5′-untranslated region and intron 1 were added to each promoter fragment upstream of the luciferase reporter gene. Fragments −904/+269, 479/+269, and −170/+269 all directed higher levels of luciferase activity in transfected K562 cells (Fig. 2A). When transiently transfected into HeLa cells, none of the AHSP promoter reporter plasmids directed significant levels of luciferase expression over background compared with the control SV40 promoter plasmid (Fig. 2A).

TABLE ONE

| Oligonucleotide primers         | Sequence                                      |
|----------------------------------|-----------------------------------------------|
| AHSP GATA-1                      | 5′-TGGCCCTTGTATCTTTCACC-3′                    |
| Sense                            |                                              |
| Antisense                        | 5′-GGTAGAAAGATAACAAGGCA-3′                    |
| Mutant AHSP GATA-1               | 5′-TGGCCCTTGTATCTTTCACC-3′                    |
| Sense                            |                                              |
| Antisense                        | 5′-GGTAGAAAGATAACAAGGCA-3′                    |
| Control GATA-1                   | 5′-GGTAGAAAGATAACAAGGCA-3′                    |
| Sense                            |                                              |
| Antisense                        | 5′-CGTATGGGCCCTTATCTTTCACCACCC-3′             |
| Wild-type AHSP Oct-1             | 5′-ACGATATGTAAATTCTA-3′                       |
| Sense                            |                                              |
| Antisense                        | 5′-TAGAATTTACATATCTGG-3′                      |
| Mutant AHSP Oct-1                | 5′-ACGATCTGCAACCTCTA-3′                       |
| Sense                            |                                              |
| Antisense                        | 5′-TAGGGTTCGATCTGG-3′                         |
| Control Oct-1                    | 5′-TGCTGAATGCAAATCTAAGA-3′                    |
| Sense                            |                                              |
| Antisense                        | 5′-TTCTAGTGATTTCGACATTTG-3′                   |

FIGURE 4. Electrophoretic gel mobility shift assays of the Oct-1 site of the human AHSP gene promoter. Gel mobility shift assays using a probe corresponding to the Oct-1 binding site of the human AHSP promoter identified by in vitro footprinting were performed using K562 nuclear extracts. Wild-type and mutant AHSP Oct-1 probes and a control Oct-1 probe were used. An Oct-1 antibody was added to the reaction mixtures where indicated.
Both AHSP and control Oct-1 complexes were effectively competed by an excess of unlabeled AHSP Oct-1 oligonucleotide, unlabeled control Oct-1 oligonucleotide, and Oct-1 antisera (Fig. 4). When a double-stranded oligonucleotide with mutation of the AHSP Oct-1 consensus sequence (ATGTAAAT to CTGCAACC) (20, 21) was used in EMSA, complex formation was abolished (Fig. 4). These data indicate that Oct-1-binding proteins bind in vitro to the AHSP gene promoter.

GATA-1 and Oct-1 Are Major Activators of the Human Erythroid AHSP Gene Promoter—To assess the relative importance of the −98 to −104 GATA-1 and +238 to +250 Oct-1 transcription factor binding sites in promoter function, mutations were introduced into these sites individually and in combination in the minimal −170/+269 AHSP promoter/luciferase reporter plasmid. Wild-type and mutant plasmids were transiently transfected into K562 cells and luciferase activity assayed after 24 h (Fig. 2B). Mutation of the GATA-1 consensus sequence (site 1, GATA to GTTA) (19) decreased luciferase activity by 33% compared with wild type. Mutation of the Oct-1 site in intron 1 (ATGTAAAT to CTGCAACC) (20, 21) reduced promoter activity by 60%. Mutation of both the GATA-1 site and the Oct-1 site reduced promoter activity to background.

Transactivation of the AHSP Gene Promoter by GATA-1 in Heterologous Cells—The −170/+269 human AHSP gene minimal promoter luciferase plasmid was transiently transfected into HeLa cells. Luciferase activity was identical to background (Fig. 5). Cotransfection of 1 μg of this AHSP gene promoter plasmid with increasing amounts of a GATA-1 cDNA expression plasmid (22) resulted in increasing luciferase activity with increasing amounts of GATA-1 plasmid (Fig. 5). However, cotransfection of 1 μg of the −170/+269 AHSP gene promoter plasmid with increasing amounts of an Oct-1 cDNA expression plasmid (IMAGE 4622256 in pcDNA3) did not result in any change in luciferase activity with increasing amounts of Oct-1 plasmid (Fig. 5).
All five AHSP/A-γ-globin transgenic lines expressed γ-globin mRNA in adult reticulocytes (TABLE TWO and Fig. 7B). The mean level of A-γ-globin compared with the level of murine α-globin mRNA was ~0.046 ± 0.5. There was no correlation between transgene copy number and mRNA level, indicating the level of transgene mRNA was influenced by position effects.

To determine the distribution of human γ-globin protein in the red cells of transgenic animals, an anti-human γ-globin monoclonal antibody was used in fluorescence-activated cell sorting analyses. The pattern of γ-globin expression in erythrocytes of two transgenic lines was uniform, i.e. present in 100% of cells (TABLE THREE, Fig. 7C). In two other transgenic lines, human γ-globin expression was variegated. In the fifth line, expression was too low for accurate analyses.

The level of transgene expression was examined in tissues of mice from two transgenic lines perfused with saline immediately prior to sacrificing. RNase protection detected γ-globin mRNA only in reticulocytes, adult bone marrow, and spleen but not in nonerythroid tissues (TABLE THREE).
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| TABLE TWO |
| --- |
| **Expression of γ-globin in transgenic mice** |
| Transgenic line (copy no.) | Transgene copy number | γ-Globin mRNA/murine α-globin mRNA | γ-Globin mRNA/murine α-globin mRNA per copy | Uniform or variegated γ-globin protein expression in erythrocytes |
| A | 11 | 0.487 ± 0.016 | 0.043 | Variegated |
| B | 3 | 0.066 ± 0.003 | 0.222 | Uniform |
| C | 9 | 0.049 ± 0.003 | 0.005 | N/A* |
| D | 6 | 0.124 ± 0.002 | 0.021 | Variegated |
| E | 1 | 0.14 ± 0.009 | 0.14 | Uniform |
| **Average** | | 0.0462 ± 0.005# |

* Expression too low to detect.
# p² = 0.32, p = not significant.

| TABLE THREE |
| --- |
| **Pattern of γ-globin mRNA expression in tissues of AHSP/γ-globin transgenic mice** |
| Transgenic line (copy no.) | Tissue | γ-Globin mRNA/murine α-globin mRNA | γ-Globin mRNA/murine α-globin mRNA per copy |
| Line B (3) | | | |
| Marrow | 0.082 ± 0.003 | 0.027 |
| Spleen | 0.064 ± 0.007 | 0.022 |
| Brain | No expression |
| Heart | No expression |
| Kidney | No expression |
| Skeletal muscle | No expression |
| Liver | No expression |
| Lung | No expression |
| Thymus | No expression |
| Line E (1) | | | |
| Marrow | 0.13 ± 0.005 | 0.13 |
| Spleen | 0.19 ± 0.010 | 0.19 |
| Brain | No expression |
| Heart | No expression |
| Kidney | No expression |
| Skeletal muscle | No expression |
| Liver | No expression |
| Lung | No expression |
| Thymus | No expression |

DISCUSSION

There are numerous consensus GATA-1 binding sites in the AHSP gene, including 5 in the minimal −170/+269 promoter region, 3 in the 5′-flanking region and 2 in intron 1. In the AHSP minimal promoter, the functionally important GATA-1 site at −98 to −104 and a second site at −47 to −52 are conserved between mouse and man. The −47 to −52 GATA-1 site was not protected in in vitro footprinting (Fig. 3) and did not bind GATA-1 in electrophoretic mobility shift assays (not shown). Transactivation of the AHSP promoter by GATA-1 in nonerythroid cells is consistent with the observation that AHSP is induced by GATA-1.

The second region protected in DNase I footprinting corresponded to an Oct-1 consensus site in the AT-rich region of AHSP intron 1. EMSA and transfection assays demonstrated the functional importance of Oct-1 in vitro. Oct-1 is a ubiquitously expressed member of the POU domain family of transcription factors known to regulate numerous tissue-specific and ubiquitous genes as transcriptional activators or repressors (20, 21). Recent gene ablation studies have demonstrated a critical role for Oct-1 in embryonic development and erythropoiesis (26). E12.5 fetal livers from Oct-1-deficient embryos demonstrated decreased numbers of TER-119-positive cells and decreased amounts of β-globin mRNA in erythroid cells.

The minimal AHSP promoter utilized in the transgenic mouse studies demonstrated that it contains the sequences necessary for erythroid-specific expression of AHSP. However, the lack of correlation between transgene mRNA levels and transgene copy number and the variegated expression seen in some lines suggesting that additional elements are necessary for authentic expression of the AHSP gene.

AHSP deficiency worsened the phenotype in β-thalassemic mice, leading to the suggestion that AHSP could be a modifier gene influencing the phenotype of human β-thalassemia syndromes (1, 6), which are marked by clinical heterogeneity (27). In a study of 120 thalassemia patients with hemoglobin E-thalassemia of varying clinical severity from Thailand, no mutations were identified in the exons of the AHSP gene or in the 5′-flanking DNA (28). Similar results were found in a population of β-thalassemic patients from Italy (29). However, in separate preliminary reports (30, 4), thalassemic patients with discordant phenotypes were found to have decreased AHSP mRNA expression in reticulocytes and cultured erythroid precursors as determined by real-time, reverse transcription polymerase chain reaction. In one discordant thalassemic patient with decreased reticulocyte AHSP mRNA, reduced amounts of AHSP protein were observed in reticulocytes and cultured erythroid cells (4). Similar to the β-globin gene mutations causing thalassemia, it is possible that there are genetic differences at the AHSP locus, or in genetic or epigenetic regulators, that influence AHSP gene expression. We suggest that the −170/+269 region be examined in thalassemia patients with varying severity, especially those with decreased AHSP.

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