Sequences Downstream of the Erythroid Promoter Are Required for High Level Expression of the Human α-Spectrin Gene*

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Ellice Y. Wong‡, Jolinta Lin§, Bernard G. Forget¶, David M. Bodine§, and Patrick G. Gallagher‡

From the ‡Department of Pediatrics and ¶Internal Medicine, Yale University School of Medicine, New Haven, Connecticut 06520-8021 and §Hematopoiesis Section, National Human Genome Research Institute, National Institutes of Health, Bethesda, Maryland 20892

α-Spectrin is a membrane protein critical for the flexibility and stability of the erythrocyte. We are attempting to identify and characterize the molecular mechanisms controlling the erythroid-specific expression of the α-spectrin gene. Previously, we demonstrated that the core promoter of the human α-spectrin gene directed low levels of erythroid-specific expression only in the early stages of erythroid differentiation. We have now identified a region 3′ of the core promoter that contains a DNase I hypersensitive site and directs high level, erythroid-specific expression in reporter gene transfection assays. In vitro DNase I footprinting and electrophoretic mobility shift assays identified two functional GATA-1 sites in this region. Both GATA-1 sites were required for full activity, suggesting that elements binding to each site interact in a combinatorial manner. This region did not demonstrate enhancer activity in any orientation or position relative to either the α-spectrin core promoter or the thymidine kinase promoter in reporter gene assays. In vivo studies using chromatin immunoprecipitation assays demonstrated hyperacetylation of this region and occupancy by GATA-1 and CBP (cAMP-response element-binding protein (CREB)-binding protein). These results demonstrate that a region 3′ of the α-spectrin core promoter contains a GATA-1-dependent positive regulatory element that is required in its proper genomic orientation. This is an excellent candidate region for mutations associated with decreased α-spectrin gene expression in patients with hereditary spherocytosis and hereditary pyropoikilocytosis.

Spectrin, the most abundant protein of the erythrocyte membrane skeleton, exists in the erythrocyte as a heterodimer of two homologous proteins, α-spectrin and β-spectrin (1, 2). α- and β-spectrin are composed primarily of 106-amino acid repeats that fold into three antiparallel α-helices connected by short non-helical segments (3–7). αβ-Spectrin heterodimers self-associate to form tetramers and higher order oligomers, forming a lattice-like structure that provides stability and deformability to the erythrocyte membrane (8–12). In the erythrocyte, spectrin functions include maintenance of cellular shape, regulation of the lateral mobility of integral membrane proteins, and provision of structural support for the lipid bilayer (2, 13).

Quantitative and qualitative disorders of spectrin have been associated with abnormalities of erythrocyte shape including hereditary spherocytosis, hereditary elliptocytosis, and hereditary pyropoikilocytosis (12, 14–19). Structural abnormalities of α spectrin in the region of the αβ self-association site are the most common defects associated with hereditary elliptocytosis and hereditary pyropoikilocytosis. However, in many patients with α-spectrin-linked hereditary spherocytosis and hereditary pyropoikilocytosis, the precise genetic defect(s) is unknown. Studies suggest that these patients have a defect in α-spectrin mRNA accumulation, which has been termed a “thalassemia-like” defect (20, 21).

The identification and characterization of the regulatory elements that control α-spectrin gene expression has important implications for understanding the pathogenesis of α-spectrin-linked hemolytic anemia and erythrocyte membrane protein biosynthesis and assembly. In splenic erythroblasts isolated from mice early after Friend virus infection, there is marked synthesis of spectrin with a significant excess of α-spectrin over β-spectrin (22). Studies in avian and rat cells have shown that the increased α-spectrin synthesis in early erythropoiesis is controlled at the transcriptional level (23–25). However, the molecular mechanisms that regulate the erythroid tissue-specific or developmental stage-specific expression of α-spectrin, including the mechanisms that control the increase in α-spectrin gene transcription to high levels during the early stages of erythropoiesis, are unknown. Our previous studies demonstrated that the core promoter of the human α-spectrin gene directed very low levels of erythroid-specific expression only in the early stages of erythroid development, indicating that elements outside the core α-spectrin gene promoter are required for high level erythroid expression (26).

This report describes the identification and characterization of a region 3′ of the α-spectrin gene promoter that contains a DNase I hypersensitive site and directs high level, erythroid-specific expression in transient and stable reporter gene/transfection assays. This region contains two functional GATA-1 sites, both required for full activity. In transfection assays this region did not display characteristics of a classical enhancer. Chromatin immunoprecipitation assays demonstrated hyperacetylation of this region and occupancy of GATA-1 and CBP.3 These results demonstrate that a region 3′ of the α-spectrin promoter positioned in its proper genomic orientation is required for high level

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† To whom correspondence should be addressed: Dept. of Pediatrics, Yale University School of Medicine, 333 Cedar Street, P. O. Box 208064, New Haven, CT 06520-8064, Tel.: 203-688-2896; Fax: 203-785-6974; E-mail: patrick.gallagher@yale.edu.

3 The abbreviations used are: CBP, cAMP-response element-binding protein (CREB)-binding protein; ChIP, quantitative chromatin immunoprecipitation.
erythroid-specific, α-spectrin gene expression. This is an excellent candidate region for mutations associated with decreased α-spectrin gene expression in patients with hereditary spherocytosis and hereditary pyropoikilocytosis.

**EXPERIMENTAL PROCEDURES**

**DNase I Hypersensitive Site Mapping—**DNase I hypersensitive site mapping was performed as described with minor modifications (27, 28). Approximately $1 \times 10^6$ logarithmically growing K562, SH SY5Y (human, neuroblastoma), or Jurkat (human, T lymphocyte) cells were collected by centrifugation, washed in cold phosphate-buffered saline, and resuspended in 14 ml of ice cold RSB (10 mM Tris, pH 7.5, 10 mM NaCl, 3 mM MgCl$_2$) to which 375 μl of 10% Nonidet P-40 was added dropwise with gentle mixing. The nuclei were pelleted by gentle centrifugation and resuspended in ~1.5 ml of ice-cold RSB. 200 μl of nuclei were placed in tubes containing increasing amounts of DNase I (0–4.0 μg/ml) in a volume of 30 μl. After a 37 °C incubation for 10 min, 230 μl of stop buffer (1% SDS, 20 mM Tris, pH 7.5, 600 mM NaCl, 10 mM EDTA, 500 μg/ml proteinase K) was added. The DNA was digested at 37 °C overnight before extraction with phenol, phenol/CHCl$_3$, and CHCl$_3$ followed by ethanol precipitation. DNA was digested with HindIII for Southern blot analysis using a 600-bp PstI/BglII fragment containing the α-spectrin core promoter as a probe. For fine mapping the migration of the band generated by DNase I and HindIII digestion was compared with the migration of bands generated by the digestion of high molecular weight K562 DNA digested with HindIII and either PstI, PvuII, EcoRI, MfeI, NheI, or EcoRV.

**Preparation of Promoter-Reporter Plasmids for Transfection Assays—**A 794-bp human α-spectrin gene promoter fragment, −793 to +1 (26), subcloned upstream of the firefly luciferase reporter gene in the plasmid pGL2B (Promega Corp.) was used as the parent plasmid. DNA fragments corresponding to exon 1’, intron 1’, and exon 1’ + intron 1’ were amplified by PCR using primers A+B, C+D, and A+D, respectively (Table I), and subcloned between the 3’ end of the α-spectrin promoter and the luciferase reporter gene. Serial truncations of these 793-bp fragment in the pGL2B plasmid were constructed by PCR amplification using the primers described in Boulanger et al. (26). Mobility of all test plasmids was confirmed by sequencing.

**Transfection Analyses—**Transient transfections were performed exactly as described (26). Stable transfections were performed as described (29) with minor modifications. 10$^7$ K562 cells were transfected by electroporation with a single pulse of 300 V at 960 microfarads with 500 μg of SalI-linearized test plasmid and 1.0 μg of poly(dI-dC). After digestion with DNase I, samples were electrophoresed in 6% denaturing polyacrylamide gels, and DNase I hypersensitive site (HS) was denoted by the boxed potential DNA-protein binding sites are underlined. The initiator motif is boxed. The PvuII site utilized in DNase I hypersensitive site fine mapping is underlined.

**Quantitative Chromatin Immunoprecipitation (ChIP) Assay—**Anti-diacetylated histone H3 (06-599) and anti-tetraacetylated histone H4 (06-866) antibodies were obtained from Upstate Biotechnology Corp. (Lake Placid, NY). Anti-GATA-1 antibody (N6) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-CBP antibody (A-22) was obtained from Santa Cruz. ChIP analysis was performed as described by Andrews and Faller (30) or Dignam et al. (31).

**In Vitro DNase I Footprinting—**Probes for in vitro DNase I footprinting were produced by PCR amplification using an α-spectrin genomic fragment, β2301 (wild type probe) (32), or on an α-spectrin gene plasmid with mutations of the GATA-1 sites in both exon 1’ and intron 1’ (Fig. 6, mutant exon 1’ + mutant intron 1’) as template and primers E and F (Table I). One oligonucleotide, either E or F, was 5' end-labeled with [γ$^32$P]ATP using polynucleotide kinase before use in PCR. Reaction mixtures 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, and the gels were dried and subjected to autoradiography.

**Gel Mobility Shift Analyses—**Binding reactions were carried out as described (26). Competitor oligonucleotides were added at molar excesses of 100-fold. Antibodies to GATA-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-CBP antibody (A-22) was obtained from Santa Cruz. ChIP analysis was performed as described by Andrews and Faller (30) or Dignam et al. (31).
scribed (33, 34). After formaldehyde fixation, chromatin was fragmented by sonication 10 times for 10 s each. Extracts were precleared with protein G-Sepharose, antibody was added, and incubated overnight at 4 °C. After elution and extraction, immunoprecipitated DNA was analyzed by quantitative real-time PCR (iCycler, Bio-Rad). PCR primers A and D (Table I) amplify the 183-bp 5′/H11032/untranslated region. Samples from at least three independent immunoprecipitations were analyzed. SYBR green fluorescence in 25-μl PCR reactions 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 agarose gel electrophoresis with ethidium bromide staining. Parallel controls for each experiment included samples of no chromatin, no antibody, nonimmune rabbit IgG, and rabbit nonimmune serum.

RESULTS

Identification of a DNase I Hypersensitive Site Downstream from the Core α-Spectrin Gene Promoter—The human α-spectrin gene is >120 kilobases and is encoded by 52 exons. This large size makes a systematic search for regulatory elements difficult by functional assays. To identify important nonpromoter-related regulatory sequences, we examined the DNase I hypersensitivity of the α-spectrin gene in the 5′ and 3′ regions as well as in the first few introns using K562 cell nuclei. A hypersensitive site was identified in a 3-kilobase HindIII fragment that mapped 3′ of the core α-spectrin gene promoter (Fig. 1A). Fine mapping localized this hypersensitive site to a 183-bp region 3′ of a PvuII site (Fig. 1, B and C). As a positive control, the same preparation of K562 DNA was digested with varying concentrations of DNase I followed by digestion with EcoRI and hybridization to a human BamIII/PvuII γ-globin probe as described (35). A hypersensitive site in the γ-globin promoter generated a new band at 1.5 kilobases, as previously shown by Groudine et al. (35) using K562 cell nuclei (not shown). Digestion of the same preparation of K562 DNA with varying concentrations of DNase I followed by digestion with SacI and

FIG. 2. Activity of the α-spectrin gene erythroid promoter and downstream 183-bp region in erythroid cells in transient transfection assays. Plasmids containing the α-spectrin gene promoter and the 183-bp region encoding exon 1′ and intron 1′ inserted upstream of the firefly luciferase gene were transfected into K562 and 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 the means ± S.D. of at least six independent transfection experiments. A, truncations of the α-spectrin gene promoter with and without the downstream sequence. B, addition of the downstream region corresponding to exon 1′ + intron 1′, exon 1′, intron 1′, and exon 1′ + intron 1′ in the reverse orientation.
hybridization to a human keratin 14 probe (36) demonstrated that the DNase I hypersensitivity of the \( \beta \)-spectrin gene and the G\( \beta \)-globin gene is not the result of general DNase I digestion of the DNA (not shown). When DNase I hypersensitivity experiments were performed with nuclei from the nonerythroid cells SH SY5Y (neuroblastoma) and Jurkat (T lymphocyte) and the same \( \beta \)-spectrin probe, no DNase I sensitivity was observed (not shown).

The Region Corresponding to the Hypersensitive Site Contains Binding Sites for GATA-1- and AP-1-binding Proteins—We inspected the DNA sequence of the region corresponding to the DNase I hypersensitive site. Consensus binding sequences for GATA-1 (two sites) and AP1 binding proteins (one site), both important for expression in other erythroid genes, were identified (Fig. 1D). This 183-bp region encodes an untranslated exon (exon 1', 61 bp) and an alternatively spliced, untranslated intron (intron 1', 122 bp) (32).

Addition of the 183-bp Region to an \( \beta \)-Spectrin Gene Promoter Fragment Significantly Increases Promoter Expression in Erythroid Cells—The 183-bp region was placed immediately 3' of the \( \alpha \)-spectrin gene promoter in its proper genomic orientation upstream of a luciferase reporter gene. This plasmid, p794/183, was transiently transfected into K562 cells. The relative luciferase activity was determined 48 h after transfection and compared with the activity obtained with the core \( \beta \)-spectrin promoter plasmid (p794 (26)), a negative control promoterless luciferase plasmid (pGL2B), and a positive control with the luciferase reporter gene under control of the SV40 early promoter (pGL2P). As shown in Fig. 2, the \( \alpha \)-spectrin gene promoter + downstream sequence plasmid, p794/183, directed 10-fold high level expression of the luciferase reporter gene in erythroid cells than the promoter alone.

**Table 1**

| Oligonucleotide primers | 5' | A | G | T | C |
|-------------------------|----|---|---|---|---|
| A                       | 5'-GCCGGATCCATGTCTTCTAAAGATAATGTCGATTG-3' |
| B                       | 5'-GGGCTCGAGCTCTTGCTTGGTCCTAGAATC-3' |
| C                       | 5'-GCCGGATCCGTTTTTTTTTTTTCCCCACATACTTAACGGTTT-3' |
| D                       | 5'-GGGGGATCCGGTTTAGAACCTGGCAAGATAA-3' |
| E                       | 5'-GCCGGTACCGTCACCCAGTATCTGTAAAAC-3' |
| F                       | 5'-GGGCTCGAGTTTTCCTAAAGGTTTAGAACC-3' |
| G                       | 5'-CTTCTAAAGATAATGTCGAT-3' |
| H                       | 5'-ATCGACATTATCTTTAGAAG-3' |
| I                       | 5'-CACATTTTATCTTGCCAGGT-3' |
| J                       | 5'-ACCTGGCAAGATAAAGTGTG-3' |
| K                       | 5'-GTCGGGTAAGAAGGATAAGGCCCCATCAG-3' |
| L                       | 5'-CTGATGCGGCTTATCTTCTTACCCACC-3' |
| M                       | 5'-CAACGTTCTTTGGATTTCCCTC-3' |
| N                       | 5'-CTCCTGCTCATCAGCTAGTAAGG-3' |
| O                       | 5'-ACTATAAGGCCACCCCTGCTG-3' |
| P                       | 5'-CCTTTGAACCCCTGCTGCTGCTG-3' |
| Q                       | 5'-GGACCTTTGGTCCGCTGCTCT-3' |
| R                       | 5'-AGGCCTTGCCCTGAGTAAGG-3' |

**Fig. 3.** In vitro DNase I footprinting of the 183-bp region 3' of the human \( \alpha \)-spectrin promoter. In vitro DNase I footprinting of the sequence downstream of the human \( \alpha \)-spectrin gene promoter was performed with a wild type probe using K562 extracts as described in "Experimental Procedures" for details. A, exon 1. A single protected site, AAGATAA, was identified on the sense strand corresponding to a GATA-1 protein consensus binding site. B, intron 1'. A single protected site, TCTATTT, was identified on the antisense strand corresponding to a GATA-1 protein consensus binding site. C and D, in vitro DNase I footprinting of the same downstream region performed with a probe with both exon 1' and intron 1' GATA-1 sites mutated using K562 extracts. Protected sites are not observed in the region of the mutated GATA-1 sequences.
The addition of the 183-bp region to deletion mutants of the core \( \alpha \)-spectrin gene promoter directed significantly higher levels of expression than the corresponding promoter fragment alone (Fig. 2A). These sequences even conferred activity to a minimal \( \alpha \)-spectrin gene promoter fragment, p44/183, which is inactive in the absence of this element.

The \( \alpha \)-Spectrin Gene Promoter Requires Both Exon 1 and Intron 1 for Full Expression in Erythroid Cells—To determine the relative importance of exon 1 and intron 1 in increasing expression directed by the \( \alpha \)-spectrin gene promoter, luciferase reporter plasmids with exon 1, intron 1, and exon 1 plus intron 1 in forward (genomic) and reverse orientations were transfected into K562 and HeLa cells. In K562 cells, plasmids with exon 1 plus intron 1 in reverse orientation (p794/183Rev) directed low levels of expression at levels comparable with those directed by the promoter alone (p794, \( p \) = not significant, unpaired \( t \) test) or the promoter plus exon 1 (p794/Exon, \( p \) = not significant, unpaired \( t \) test) (Fig. 2B). Promoter plus intron 1 plasmids (p794/Intron) directed higher levels of expression, approximately half that directed by p794/183 (\( p = 0.0046 \), unpaired \( t \) test). In HeLa cells there was no change in levels of luciferase expression of any of the plasmids. These results suggest that exon 1 and intron 1 in their appropriate genomic orientation are required for full expression in erythroid cells.

The 183-bp Region of the \( \alpha \)-Spectrin Gene Contains Binding Sites for GATA-1- and AP-1-binding Proteins—To identify sites for DNA-binding proteins within the 183-bp region, DNase I footprinting analysis with nuclear extracts from K562 cells was performed. Two footprints were observed. One site was in exon 1 on the sense strand (5'-AAGATAA-3') (Fig. 3A), and the other was in intron 1 on the antisense strand (5'-AGATAAA-3') (Fig. 3B). Both contained consensus binding sequences for the erythroid transcription factor GATA-1. The footprinted region in intron 1 did not extend into the AP-1 consensus sequence.

GATA-1 Binds Both Exon 1 and Intron 1 GATA Sites in the 183-bp Region in Electrophoretic Mobility Shift Assays—To determine which nuclear proteins bound to the GATA-1 sites present in exon 1 and intron 1, double-stranded oligonucleotides containing the corresponding \( \alpha \)-spectrin promoter GATA-1 sequences (exon 1 G + H; intron 1 I + J; Table I) or control GATA-1 sequences (K + L; Table I) (37) were prepared and used in gel shift analyses. A single complex was observed in K562 (erythroid) extracts when probes corresponding to either site were used (Figs. 4, A and B). This complex migrated at the same location as a control oligonucleotide containing a GATA-1 consensus sequence. This species was effectively competed both by an excess of unlabeled homologous oligonucleo-
tide and by an excess of unlabeled control GATA-1 oligonucleotide (not shown). The inclusion of GATA-1 antisera abolished most or all of the DNA binding.

**Mutation of the GATA-1 Sites in the 183-bp Region Disrupts GATA-1 Binding in Vitro—**

To further assess the ability of nuclear proteins to bind the \( \alpha \)-spectrin GATA-1 sites in vitro, DNase I footprinting and gel mobility shift assays were performed using probes with the GATA-1 sites mutated (GATA to GTTA) (38). For \emph{in vitro} DNase I footprinting, mutations were introduced into both exon 1' and intron 1' GATA-1 sites of the \( \alpha \)-spectrin gene promoter + the 183-bp probe described above. When footprinting was performed with this double mutant

**Table II**

Activity of the \( \alpha \)-spectrin gene erythroid promoter and downstream 183-bp region in stably transfected K562 cells

| Plasmid                                      | Number of stably transfected lines | Normalized luciferase activity per copy number |
|----------------------------------------------|-----------------------------------|-----------------------------------------------|
| pGL2B (promoterless)                        | 18                                | 1                                             |
| pGL2P (SV40 promoter)                       | 11                                | 14.8 ± 3.1                                    |
| p794 (\( \alpha \)-spectrin promoter)        | 9                                 | 27.67 ± 3.1                                   |
| p794/183 (\( \alpha \)-spectrin promoter + downstream element) | 10                                | 86.2 ± 15.5                                   |
| Mutant exon 1' + mutant intron 1' (\( \alpha \)-spectrin promoter + downstream element with both GATA-1 sites mutated) | 10                                | 1.7 ± 0.3                                     |

**Fig. 6.** GATA-1 transactivates the human \( \alpha \)-spectrin gene promoter in heterologous cells when the 183bp region is included. \( \alpha \)-Spectrin gene promoter/luciferase reporter plasmids without the 183-bp region (A), with the 183-bp region (B), with exon 1' (C), with intron 1' (D), or with the GATA-1 sites (E) in exon 1' and intron 1' of the 183-bp region mutated were cotransfected with increasing amounts of a GATA-1 cDNA expression plasmid into HeLa cells (see "Methods" for details). Dose-dependent activation of the \( \alpha \)-spectrin gene promoter was not observed when the 183-bp region was not included or the GATA-1 sites in exon 1' and intron 1' of the 183-bp region were mutated.
GATA-1 probe and K562 cell nuclear extracts, no protected regions at the mutant GATA-1 sites were observed (Fig. 3C). When oligonucleotides with mutation of the consensus GATA-1 binding sequences (GATA to GTTA) (38) were used in gel mobility shift assays, complex formation was nearly or completely abolished (Fig. 4, C and D). These data indicate that GATA-1 binds to the exon 1′ and intron 1′ sites 3′ of the a-spectrin gene promoter in vitro.

**Mutation of the 183-bp Region GATA-1 Sites Significantly Decreases Promoter Function**—To assess the relative importance of these GATA-1 binding sites in promoter function, mutations were introduced into the GATA-1 sites protected in DNase I footprinting experiments, both individually and in combination. Mutation of the exon 1′ GATA-1 site (GATA to GTTA) (37) decreased activity by approximately half in transiently transfected K562 cells (Fig. 5). Mutation of the intron 1′ GATA-1 site in a similar manner (GATA to GTTA) reduced promoter activity by 66% (Fig. 5). When a reporter plasmid with mutations of both GATA-1 sites was transfected into K562 cells, promoter activity was reduced to background levels (Fig. 5). These results provide further evidence that the GATA-1 elements in the downstream element exert important effects on a-spectrin gene expression.

**A-Spectrin Gene Expression**

### Table: Relative Luciferase Activity

| A | Relative Luciferase Activity |
|---|-----------------------------|
| ASp Promoter | 71 ± 4 |
| ASp Promoter Exon Intron Luc | 798 ± 58 |
| 5′ Exon Intron 3′ ASp Promoter | 25 ± 4 |
| 5′ Exon Intron 3′ ASp Promoter Luc | 21 ± 1 |
| 3′ ASp Promoter | 13 ± 1 |
| 3′ ASp Promoter Luc 5′ Exon Intron 3′ | 12 ± 0 |

### B

| Relative Luciferase Activity |
|-----------------------------|
| TK Promoter | 196 ± 0.4 |
| TK Promoter 5′ Exon Intron 3′ | 217 ± 2.7 |
| TK Promoter 5′ Exon Intron 3′ | 182 ± 1.0 |
| TK Promoter Luc 3′ 5′ | 299 ± 1.1 |
| TK Promoter Luc 3′ 5′ | 122 ± 1.3 |

GATA-1 Transactivates the Human a-Spectrin Gene Promoter in Heterologous Cells When the 183-bp Region Is Present—None of the a-spectrin gene promoter-exon 1′ +/− intron 1′ fragments directed significant levels of expression of a reporter gene in HeLa cells (Fig. 2B). Co-transfection of 1 μg of the core 794-bp a-spectrin promoter plasmid without the 183-bp region with increasing amounts of a GATA-1 cDNA expression plasmid (39) into HeLa cells did not result in increased expression of luciferase (Fig. 6). Co-transfection of HeLa cells with increasing amounts of a GATA-1 cDNA expression plasmid and 1) the a-spectrin promoter + exon 1′/reporter
plasmid, 2) the α-spectrin promoter + intron 1'/reporter plasmid, or 3) the α-spectrin promoter + exon 1' + intron 1'/reporter plasmid directed dose-dependent levels of luciferase activity (Fig. 6). Co-transfection of the α-spectrin promoter + exon 1' + intron 1'/reporter plasmid with both GATA-1 sites mutated and increasing amounts of a GATA-1 cDNA expression plasmid did not result in increased expression of luciferase (Fig. 6). These results indicate that transactivation of the α-spectrin gene promoter with exon 1' and/or intron 1' in HeLa cells requires GATA-1 expression.

The 183-bp Region Does Not Act as a Classical Enhancer Element—To test if the 183-bp region functions as an enhancer

**FIG. 8.** Quantitative ChIP analysis of the 183-bp region of the α-spectrin gene. A, ethidium bromide-stained agarose gel of sonicated, deproteinized chromatin fragments used in the ChIP assay. B, standard curves of SYBR green fluorescence obtained from K562 cells using a primer pair encompassing the 183-bp region. C, representative amplification plots of preimmune serum and DNA from chromatin immunoprecipitated with anti-H3 and anti-H4 antibodies at the 183-bp region of the α-spectrin gene in K562 cells. D, dissociation plot obtained from the immunoprecipitated DNA amplicons shown in C. The single peak indicates amplification of a single amplicon.
element, the 183-bp fragment was positioned in both orientations upstream and downstream of the core α-spectrin gene promoter-luciferase reporter gene, and the corresponding plasmids were transfected into K562 cells. With all four combinations, luciferase expression was significantly reduced compared with the wild type promoter alone (Fig. 7A).

Luciferase reporter plasmids with the 183-bp fragment positioned in both orientations upstream and downstream of a heterologous thymidine kinase promoter-luciferase reporter gene were prepared. Luciferase expression was assayed after transfection of the plasmids in K562 cells. When the 183-bp fragment was placed upstream or downstream of the thymidine kinase promoter in either orientation, levels of expression were similar compared with that directed by the wild type thymidine kinase promoter without additional sequences (Fig. 7B).

Chromatin Immunoprecipitation Analysis of the 183-bp Region—Histone modifications within the 183-bp region of the α-spectrin gene were examined using a ChIP assay with anti-diacetylhistone H3 and anti-tetraacetyl histone H4 antibodies and PCR primers (A + D) that amplified across the 183-bp region. The core histones H3 and H4 were hyperacetylated in this region in chromatin from erythroid (K562) cells but not heterologous (HeLa) cells (Figs. 8 and 9A).

GATA-1 and CBP occupancy at the β-globin locus control region and β-major globin gene promoter has been shown to correlate with acetylation of histones H3 and H4 (40). Because the α-spectrin gene 183-bp region was hyperacetylated in erythroid cells and contains two functionally important GATA-1 sites, we performed ChIP using anti-GATA-1 and anti-CBP antibodies and the same PCR primers as above (Fig. 9B). These studies demonstrated that GATA-1 and CBP occupied the 183-bp region of the α-spectrin gene in chromatin from erythroid (K562) but not nonerythroid (HeLa) cells (Fig. 9C) in vivo.

DISCUSSION

Our previous studies demonstrated that the core promoter of the human α-spectrin gene directed low levels of erythroid-specific expression and only in primitive erythroid cells, indicating that elements outside the core α-spectrin gene promoter were required for high level erythroid expression (26). This is in contrast to the erythroid promoters of the ankyrin, band 3, and β-spectrin genes, which direct significantly higher levels of expression at all stages of erythroid development (41−45). This observation was surprising as previous studies had demonstrated that the α-spectrin gene is expressed at high levels in erythroid cells and that the expression was controlled at the transcriptional level (22−25). The demonstration that a region 3′ of the core α-spectrin gene promoter is required for high level erythroid expression begins to clarify the mechanisms of α-spectrin gene regulation in erythroid cells.

This 183-bp region encodes exon 1′ and an alternately spliced intron 1′, present in ~50% of α-spectrin mRNA transcripts (4, 32). To obtain full enhancement of expression, both elements need to be present placed downstream of the α-spectrin gene promoter in the appropriate genomic orientation. The primary enhancement of expression is mediated by two GATA-1 repeats present in the intron (51). In lymphoid cells a number of intragenic enhancer elements have been well characterized. The first intron of the GATA-3 gene contains a positive cis-acting element that serves as a T cell-specific enhancer (55). The interleukin-4 gene contains positive regulatory cis-acting elements in introns 1 and 2 that are dependent on GATA family members for their activity (56, 57). These elements are associated with DNase I hypersensitive sites and chromatin accessibility.

In the β-globin locus, GATA-1 occupancy, recruitment of the histone acetyltransferase CBP, histone acetylation, and globin gene expression have been correlated (40). These results sug-

![Fig. 9](image-url) The 183-bp region of the α-spectrin gene is hyperacetylated by H3 and H4 (A) and demonstrates occupancy by GATA-1 and CBP (B) in chromatin immunoprecipitation assays with K562 cells. C, in the 183-bp region of the α-spectrin gene there is no occupancy by GATA-1 or CBP in chromatin immunoprecipitation assays with HeLa cells. See “Results” for details.
gested that one role of GATA-1 is to establish an erythroid-specific pattern of histone acetylation at the β-globin locus. It is possible that a similar mechanism exists at the downstream α-spectrin gene GATA-1 binding sites. This is supported by the presence of functional GATA-1 sites, GATA-1 and CBP occupancy, and histone acetylation in this region. Time course experiments (39, 58, 59) will provide additional insight into this question. It is possible that other GATA-1 target genes facilitate recruitment of acetyltransferases to the α-spectrin gene downstream region or that acetyltransferases other than CBP are recruited to this region. The opening of compacted chromatin at the α-spectrin locus by GATA family transcription factors via histone binding and not by classical chromatin remodeling complexes, as shown in vitro (60, 61), is also possible.

In the inherited hemolytic anemia recessive hereditary spherocytosis and hereditary pyropoikilocytosis, there are thalassemia-like defects of α-spectrin synthesis that, when co-inherited with a structural α-spectrin mutations, produce a more severe phenotype (for review, see Refs. 14 and 62). These defects are characteristic by reduced α-spectrin mRNA levels and diminished α-spectrin synthesis. The molecular basis of this production-defective α-spectrin allele is unknown. This region downstream from the core α-spectrin gene promoter is an excellent candidate region for mutations that could cause decreased α-spectrin synthesis.

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