Erythroid Expression of the Human $\alpha$-Spectrin Gene Promoter Is Mediated by GATA-1- and NF-E2-binding Proteins*

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$\alpha$-Spectrin is a highly expressed membrane protein critical for the flexibility and stability of the erythrocyte. Qualitative and quantitative defects of $\alpha$-spectrin are present in the erythrocytes of many patients with abnormalities of red blood cell shape including hereditary spherocytosis and elliptocytosis. We wished to determine the regulatory elements that determine the erythroid-specific expression of the $\alpha$-spectrin gene. We mapped the 5' end of the $\alpha$-spectrin erythroid cDNA and cloned the 5' flanking genomic DNA containing the putative $\alpha$-spectrin gene promoter. Using transfection of promoter/reporter plasmids in human tissue culture cell lines, in vitro DNAse I footprinting analyses, and gel mobility shift assays, an $\alpha$-spectrin gene erythroid promoter with binding sites for GATA-1- and NF-E2-related proteins was identified. Both binding sites were required for full promoter activity. In transgenic mice, a reporter gene directed by the $\alpha$-spectrin promoter was expressed in yolk sac, fetal liver, and erythroid cells of bone marrow but not adult reticulocytes. No expression of the reporter gene was detected in nonerythroid tissues. We conclude that this $\alpha$-spectrin gene promoter contains the sequences necessary for low level expression in erythroid progenitor cells.

*Spectrin, the most abundant protein of the erythrocyte membrane skeleton, is composed of two structurally similar but nonidentical proteins, $\alpha$- and $\beta$-spectrin, encoded by separate genes (1, 2). $\alpha$- and $\beta$-spectrin are composed primarily of homologous 106-amino acid repeats that fold into three antiparallel $\alpha$-helices connected by short nonhelical segments (3–7). $\alpha$- and $\beta$-spectrin combine to form heterodimers, which in turn self-assemble to form tetramers and higher order oligomers to form a lattice-like structure that is critical for erythrocyte membrane stability, as well as erythrocyte shape and deformability (8–12). In the red cell, spectrin maintains cellular shape, regulates the lateral mobility of integral membrane proteins, and provides structural support for the lipid bilayer (2, 13). Quantitative and qualitative disorders of $\alpha$-spectrin have been associated with abnormalities of erythrocyte shape including hereditary spherocytosis, elliptocytosis, and pyropoikilocytosis (12, 14–19).

In erythropoiesis, differentiation of early erythroid progenitor cells into morphologically defined erythroblasts is associated with significant changes in the synthesis and expression of membrane proteins. In splenic erythroblasts isolated from mice early after Friend virus infection, there is marked synthesis of spectrin with a significant excess of $\alpha$-spectrin over $\beta$-spectrin (20, 21). However, at this stage, only a small fraction of this newly synthesized spectrin is incorporated into the membrane skeleton (21). During terminal differentiation, $\alpha$- and $\beta$-spectrin synthesis are decreased, but a markedly increased amount of spectrin is incorporated into the membrane. Studies of erythropoiesis in avian and rat cells have shown that the increased $\alpha$-spectrin synthesis in early erythropoiesis is controlled at the transcriptional level (21–23). The molecular mechanisms that regulate the tissue or developmental stage-specific expression of $\alpha$-spectrin, including the mechanisms that control the increase in $\alpha$-spectrin gene transcription to high levels during the early stages of erythropoiesis, are unknown.

The identification and characterization of the regulatory elements that control $\alpha$-spectrin gene expression have important implications for several biological processes including the pathogenesis of $\alpha$-spectrin-linked hemolytic anemia and erythrocyte membrane protein biosynthesis and assembly. Furthermore, because $\alpha$-spectrin is synthesized in large amounts 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 therapy applications.

To provide insight into the regulation of the human $\alpha$-spectrin gene, we have identified and characterized the human $\alpha$-spectrin gene promoter. Our results demonstrate that the human $\alpha$-spectrin gene promoter requires GATA-1- and NF-E2-binding proteins to direct high level expression in erythroid cells in vitro. In transgenic mice, a reporter gene directed by the $\alpha$-spectrin promoter directed expression exclusively in erythroid progenitor cells at early stages of erythroid differentiation. These results suggest that the minimal $\alpha$-spectrin gene promoter contains the sequences necessary for low level expression in erythroid progenitor cells and that additional regulatory elements are required for late developmental stage expression of the $\alpha$-spectrin gene in erythroid cells.

EXPERIMENTAL PROCEDURES

RNA Preparation and 5' Rapid Amplification of cDNA Ends—Total RNA was prepared from human tissues 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) as described (24). 1 μg of total human fetal liver RNA was reverse-transcribed using Primer A (see Table 1) and avian myeloblastosis virus reverse transcriptase (Promega). Single-stranded oligonucleotide ligation and PCR amplification were carried out as described using Primers A + C and B + C (25, 26) (see Fig. 1). Amplification products were subcloned and sequenced.

Mapping the Transcription Initiation Site—The transcription initiation site of the α-spectrin cDNA was determined using a primer extension assay. Primer D (see Table 1) was 5’-end-labeled with [32P]ATP and T4 polynucleotide kinase and then ethanol was precipitated with 20 μg of total K562 cell RNA. The pellets were resuspended in hybridization buffer, heated to 60 °C for 90 min, and then precipitated. The pellets were resuspended, and the primer was extended with avian myeloblastosis virus reverse transcriptase at 42 °C for 1 h. The RNA was then digested with RNase A. Extension products were ethanol-precipitated, dissolved in loading buffer, and analyzed on a 6% denaturing acrylamide gel.

Genomic Cloning—A human α-spectrin cDNA fragment corresponding to the 5’ end of the coding region, α19 (see Fig. 1A) (4, 27), was used as hybridization probe to screen a human genomic DNA library. The library is a Charon 4A bacteriophage library containing fragments of genomic DNA partially digested with AluI and HaeIII with EcoRI linkers added. Selected recombinants that hybridized to α19 were purified and subcloned into pGEM-7Z plasmid vectors (Promega). Subcloned fragments were analyzed by restriction endonuclease digestion, Southern blotting, and nucleotide sequencing.

Preparation of Nuclear Extracts—Nuclear extracts were prepared from K562 and HeLa cells by hypotonic lysis followed by high salt extraction of nuclei as described by Andrews and Faller (28) or Dignam et al. (29).

DNase I Footprinting in Vitro—Probes for DNase I footprinting were produced by PCR amplification of an α-spectrin genomic fragment as template and a pair of oligonucleotides primers, E and F (see Table 1). One oligonucleotide, either E or F, 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 on 6% denaturing polyacrylamide gels, and the gels were dried and subjected to autoradiography.

Preparation of Promoter-Reporter Plasmids for Transfection Assays—A 793-bp fragment corresponding to 5’ flanking α-spectrin genomic DNA was amplified using primers G and H (see Table 1), which contain sites for KpnI and XhoI, respectively. The 793-bp amplification product was digested with KpnI and XhoI and then subcloned upstream of the firefly luciferase reporter gene in the plasmid pGL2B (Promega). Serial truncations of this 793-bp fragment in the pGL2B plasmid were constructed using PCR amplification. Integrity of all test plasmids was confirmed by sequencing.

Transient Transfection Analyses—All plasmids tested were purified using Qiagen columns or cesium chloride plasmid purification, and at least two preparations of each plasmid were tested in triplicate. 107 K562 cells were transfected by electroporation with a single pulse of 300 V at 960 microfarad with 20 μg of test plasmid and 0.5 μg of pCMVβ, a mammalian reporter plasmid expressing β-galactosidase driven by the human cytomegalovirus immediate early gene promoter (Clontech) (30). 105 HeLa cells were transfected with 2.0 μg of test plasmid and 0.25 μg of the pCMVβ plasmid by lipofection using 4 μl of LipofectAMINE (Invitrogen). Twenty-four h after transfection, cells were harvested and lysed, and the activity of both luciferase and β-galactosidase activity was determined in cell extracts. All assays were performed in triplicate. Differences in transfection efficiency were determined by co-transfection with the pCMVβ plasmid.

COS cells (105) were transfected with 20 μg of the expression plasmids pMT/BKLF (31) (a kind gift of Drs. M. Crosseon and S. Orkin) or pSG5/EKLF (32) (a kind gift of Dr. J. Bieker) as described above. Forty-eight h after transfection, nuclear extracts were prepared for use in gel shift analyses.

Gel Mobility Shift Analyses—Binding reactions were carried out as described (33). Competitor oligonucleotides were added in molar excess of 100-fold. Antibodies to GATA-1, p45 NF-E2, and Sp1 were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). Reaction mixes 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 analyzed by autoradiography.

Preparation of Promoter-Reporter Plasmids for Transgenic Mice—A 793-bp α-spectrin gene promoter fragment was excised from plasmid p793 as a Small/BgII fragment and ligated into the EcoRV/BamHI sites of pSP72. A 2266-bp EcoRV/AatII fragment containing the human γ-γ-globin was excised from a pSP72 plasmid containing a 1909-bp BsaIII/HindIII fragment of the human γ-globin gene (34) and ligated into the AatII/PvuII sites of the pSP72 plasmid to generate αSp73/γ. This plasmid construct was sequenced to confirm that the α-spectrin promoter correctly fused to the γ-globin gene. Finally, a 2689-bp α-spectrin promoter–γ-globin DNA fragments were microinjected into the male pronucleus of fertilized eggs. The injected eggs were transferred into pseudopregnant CB6F1 foster mothers. Founders were identified by Southern blotting of genomic DNA obtained from tail biopsies. Founder animals were crossed to FVB/N mice for propagation.

Transgenic Copy Number Analysis—Copy number was determined by comparing the γ-globin signals from Southern blot analysis of transgenic mouse and K562 DNA using a Molecular Dynamics Phosphor-Imager. Statistical analysis of copy number and expression data was analyzed by linear regression using GraphPad Prism version 2.0 software.

Preparation of Murine RNA—Total cellular RNA was extracted from mouse tissues, including adult reticulocytes and separated splenic cells, using TRIzol reagent (Invitrogen). Splenic cells were separated into erythroid, myeloid, or lymphoid fractions using the following method. Total spleen cells were dissociated by passage through 16- and then 21-gauge needles. The single cell suspension was washed in phosphate-buffered saline with 5% fetal calf serum. Ten ml of this was used to prepare RNA by TRIzol. To the remaining 10 ml, 250 μl of TERR 119 antibody was added. The cells were incubated for 25 min on ice with mixing every 5 min. The cells were underlayed with 1 ml of phosphate-buffered saline with 10% fetal calf serum and spun at 1500 rpm for 5 min at 4 °C. The cells were resuspended in 10-ml goat and rabbit magnetic beads in 5% fetal calf serum for 30 min on ice with mixing every 5 min. The cells were applied to a magnet for 10 min. The beads and adherent cells were collected and processed directly to yield erythroid RNA. The nonadherent cells were collected and placed in a new tube to which 50 μl of anti-CD4 and 50 μl of anti-CD8 were added,
and the cells were incubated on ice for 30 min with mixing. CD4/8-positive cells were collected as above from the magnet and processed for flow cytometry. The 32P-labeled antisense RNA probe was synthesized by transcription in vitro with SP6 RNA polymerase (MAXIscript; Ambion Incorporated, Austin, TX). The probe (1/1,000,000 cpm) was hybridized to template RNA at 42 °C overnight. Template RNAs in these reactions were 10 ng of total RNA from yolk sac, fetal liver, adult reticulocyte, bone marrow, muscle, brain, heart, kidney, and spleenic cells separated into erythroid, myeloid, or lymphoid cells or 105 reticulocyte, bone marrow, muscle, brain, heart, kidney, and splenic gels.

**RESULTS**

Cloning of Chromosomal Gene, Isolation and Analysis of Recombinant Clones—Primary screening of a human genomic DNA library with the α-spectrin cDNA probe α19 (Fig. 1A) yielded multiple hybridization-positive plaques. Selected recombinants were analyzed, and one clone was identified, λ3021, that spanned ~16.5 kb of DNA containing the α-spectrin gene. A limited restriction map of this region is shown in Fig. 1B.

Mapping the Human α-Spectrin Erythroid mRNA Transcription Initiation Site and Identification of 5′ CDNA Sequences—To identify the 5′ end of the human α-spectrin cDNA, primer extension was performed using total RNA from K562 cells. These experiments identified a single transcription initiation site (Fig. 2) and predicted the presence of an additional 17 bp in the mRNA upstream of the 5′ end of the sequence obtained from cdNA cloning. These additional 17 bp of upstream 5′ untranslated sequence were obtained by 5′ rapid amplification of cdNA ends. Sequences obtained by rapid amplification of cdNA ends (Fig. 1A) were verified by comparison to corre-
The Human α-Spectrin Gene Erythroid Promoter

The 5' Flanking Genomic DNA Sequence of the Human α-Spectrin Gene Exhibits Features of an Erythroid Gene Promoter—The nucleotide sequence of the 5' flanking genomic DNA upstream of the human α-spectrin cDNA transcription start site is shown in Fig. 3. Inspection of the sequence reveals features lack consensus TATA or CCAAT sequences. Consensus sequences for a number of potential DNA-binding proteins, including GATA-1 (three sites), NF-E2, AP-1, and Sp1/CACCC-related proteins are present in the 5' flanking sequences.

An α-Spectrin Gene Promoter Fragment Is Active in Erythroid Cells—To investigate whether the region from −793 to +1 was capable of directing expression of a reporter gene in cultured mammalian cells, test plasmid p793 was transiently transfected into K562 cells. The relative luciferase activity was determined 48 h after transfection and compared with the activity obtained with pGL2B, a negative control, the promoter

responsive genomics DNA sequences (Fig. 3). The sequences around the transcription start site, GATA, TGGTC, closely match transcription initiation recognition sequences, YYA, NYYY (36). No additional ATGs were present in the 5' untranslated sequences. Taken together, these data suggest that this sequence is at or very near the 5' end of the human α-spectrin erythroid cDNA.

To determine whether nuclear proteins could bind the three GATA-1 consensus binding sites present in the human α-spectrin promoter, DNase I footprinting analysis with nuclear extracts from K562 cells was performed (Fig. 5). A single long footprint was observed. This site contains potential binding sites for GATA-1, as well as Sp1 and CACCC-related proteins, a combination shown to be adequate for expression of a minimal promoter in other erythroid-specific genes. There was minimal or no reporter gene activity of either p793 or p194 in transfected HeLa cells.

Transient transfection analysis of deletions of this α-spectrin gene erythroid promoter fragment identified a 195-bp minimal promoter fragment, p194, that directed expression of the reporter gene in erythroid cells (Fig. 4A). This minimal promoter fragment contains potential binding sites for GATA-1, as well as Sp1 and CACCC-related proteins, a combination shown to be adequate for expression of a minimal promoter in other erythroid-specific genes. There was minimal or no reporter gene activity of either p793 or p194 in transfected HeLa cells.

The α-Spectrin Erythroid Promoter Contains Binding Sites for GATA-1, NF-E2, and CACCC-related Binding Proteins—To identify binding sites for transcription factors within the core α-spectrin promoter, DNase I footprinting analysis with nuclear extracts from K562 cells was performed (Fig. 5). A single long footprint was observed. This site contains consensus binding sequences for GATA-1, NF-E2, and CACCC binding proteins.

GATA-1 Binds the α-Spectrin Gene Promoter in Vitro—To determine whether nuclear proteins could bind the three GATA-1 sites present in the α-spectrin gene promoter in vitro, double-stranded oligonucleotides containing the corresponding α-spectrin promoter GATA-1 sequences (site 1, I + J; site 2, K + L; site 3, M + N) (see Table I) or control GATA-1 sequences (O + P; see Table I) (37) were prepared and used in gel shift analyses. When oligonucleotides containing the upstream
GATA-1 sequences were used in gel shift analyses, a single retarded species was observed in K562 (erythroid) extracts when probes corresponding to either site 2 (Fig. 6) or site 3 (not shown) but not site 1 (not shown). These species 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 oligonucleotide 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 of the site 2 (Fig. 6) and site 3 probes (not shown). When oligonucleotides with mutation of the consensus GATA-1 binding sequences (GATA to GATTA) (38) corresponding to either site 2 or site 3 were used in gel mobility shift assays, complex formation was completely abolished (not shown). These data indicate that GATA-1 binds to the two downstream GATA-1 sites but not the upstream GATA-1 site of the α-spectrin gene promoter in vitro.

**Nuclear Proteins Bind the α-Spectrin Gene Promoter NF-E2 Site in Vitro**—To determine whether nuclear proteins could bind the NF-E2 consensus binding sequences in vitro, double-stranded oligonucleotides containing the corresponding α-spectrin promoter NF-E2 sequences (Q + R; see Table I) or control sequences (S + T; see Table I) (39, 40) were prepared and used in gel shift analyses. When oligonucleotides containing the footprinted NF-E2 sequences were used in gel shift analyses with K562 cells extracts, a large retarded species was observed (Fig. 7). These species migrated at the same location as a control oligonucleotide containing an NF-E2 consensus sequence. This species was effectively competed both by an excess of unlabeled homologous oligonucleotide and by an excess of unlabeled control NF-E2 oligonucleotide. The inclusion of p45 NF-E2 antisera abolished most or all of the DNA binding (Fig. 7). When a double-stranded oligonucleotide with mutation of the α-spectrin promoter consensus NF-E2 binding sequences (GCTGAGTC to TCTGAGTCA) (39, 40) was used in gel mobility shift assays, complex formation was abolished (not shown). These data indicate that NF-E2-binding proteins bind in vitro to the α-spectrin gene promoter.

**CACCC Box-binding Proteins Do Not Bind to the α-Spectrin Gene Promoter in Vitro**—The protected region obtained in DNase I footprinting included the sequence, 5’-CCACCC-3’, a consensus binding site for CACCC box-binding proteins. To determine whether the nuclear proteins Sp1, BKLF, or EKLF bind this sequence in vitro, double-stranded oligonucleotides containing the corresponding α-spectrin CACCC site sequences (U + V; see Table I) or control sequences (Sp1, W + X (41, 42); CACCC, Y + Z (32, 43); see Table I) were prepared and used in gel shift analyses. When double-stranded oligonucleotides containing the CACCC site sequences were used in gel shift analyses with K562 extracts, no complexes migrated at the same location as those obtained using control oligonucleotides containing either CACCC or Sp1 consensus sequences (not shown). To determine whether the CACCC box binding transcription factors BKLF or EKLF could bind the α-spectrin gene promoter CACCC site in vitro, gel shifts using nuclear extracts prepared from COS cells transfected with expression plasmids containing either BKLF or EKLF cDNAs and either

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**Fig. 7. Gel mobility shift assays of the NF-E2 site of the human α-spectrin gene promoter.** A. Gel mobility shift assays using α-spectrin promoter oligonucleotides corresponding to the NF-E2 consensus binding sequences and K562 nuclear extracts are shown. The radiolabeled, double-stranded oligonucleotide used in lanes 1–6 corresponds to site 3, and the radiolabeled, double-stranded oligonucleotide used in lanes 7–12 is a CACCC control. Increasing amounts of unlabeled, double-stranded oligonucleotide, site 3 (lanes 3, 4, 11, and 12), CACCC control (lanes 5 and 9), or Sp1 control (lanes 6 and 10) were added to the reactions as competitor. A p45 NF-E2 antibody was added to the reaction mixtures where indicated.

**Fig. 8. Human α-spectrin promoter and γ-globin transgene constructs and riboprobes.** A. α-spectrin promoter and γ-globin transgene. A 793-bp α-spectrin gene promoter fragment (−793 to +1) was fused to the human γ-globin gene (−4 to +1906) to create the transgene construct shown. B. hybrid human γ-globin/mouse α-globin riboprobe. An Sp6 riboprobe containing sequences for both exon 2 of the human γ-globin gene and exon 2 of the murine α-globin gene was prepared to ensure that both the human γ-globin and murine α-globin sequences are labeled to equal specific activity in ribonuclease protection assays.

**Fig. 9. Detection of α-spectrin/γ-globin mRNA in yolk sac, fetal liver, and adult reticulocytes of transgenic mice containing the α-spectrin/human γ-globin transgene.** 1.0 μg of RNA from yolk sac, fetal liver, or adult reticulocytes was hybridized to a 32P-labeled antisense riboprobe that protects exon 2 of the α-spectrin γ-globin transgene (top band) and exon 2 of the mouse α-globin gene (lower band), digested with RNase, electrophoresed in an 8% nondenaturing gel, dried, and subjected to autoradiography.
TABLE II

Developmental pattern of γ-globin mRNA expression in erythroid tissues of α-spectrin/λγ-globin transgenic mice

| Tissue       | Transgene copy | Human γ-globin RNA/mouse α-globin RNA | Human γ-globin RNA/ transgene copy |
|--------------|----------------|---------------------------------------|-----------------------------------|
| Yolk sac     | 3              | 0.15                                  | 0.05                              |
| Line B       | 3              | 0.26                                  | 0.08                              |
| Line F       | 1              | 0.04                                  | 0.04                              |
| Fetal liver  | 3              | 0.06                                  | 0.02                              |
| Line B       | 3              | 0.15                                  | 0.05                              |
| Line F       | 1              | 0.02                                  | 0.02                              |
| Adult reticulocyte |       |                                       |                                   |
| Line A       | 10             | No expression                         |                                   |
| Line B       | 3              | No expression                         |                                   |
| Line C       | 15             | No expression                         |                                   |
| Line D       | 5              | No expression                         |                                   |
| Line E       | 3              | No expression                         |                                   |
| Line F       | 1              | No expression                         |                                   |
| Line G       | 6              | No expression                         |                                   |

TABLE III

Pattern of γ-globin mRNA expression in tissues of α-spectrin/λγ-globin transgenic mice

| Transgenic line (copy number) | Tissue       | Human γ-globin RNA/mouse α-globin RNA | Human γ-globin RNA/transgene copy |
|-------------------------------|--------------|---------------------------------------|-----------------------------------|
| Line B (3)                    | Marrow       | 0.06                                  |                                   |
|                               | Brain        | No expression                         |                                   |
|                               | Heart        | No expression                         |                                   |
|                               | Kidney       | No expression                         |                                   |
|                               | Skeletal muscle | No expression       |                                   |
|                               | Liver        | No expression                         |                                   |
|                               | Lung         | No expression                         |                                   |
|                               | Thymus       | 0.02                                  |                                   |
|                               | Spleen       | 0.05                                  |                                   |
|                               | Splenic cells |                                   |                                   |
|                               | Erythroid    | 0.12                                  | 0.04                              |
|                               | Myeloid      | No expression                         |                                   |
|                               | Lymphoid     | No expression                         |                                   |
| Line D (3)                    | Marrow       | 0.03                                  |                                   |
|                               | Brain        | No expression                         |                                   |
|                               | Heart        | No expression                         |                                   |
|                               | Kidney       | No expression                         |                                   |
|                               | Skeletal muscle | No expression       |                                   |
|                               | Liver        | No expression                         |                                   |
|                               | Lung         | No expression                         |                                   |
|                               | Thymus       | 0.005                                 |                                   |
|                               | Spleen       | 0.01                                  |                                   |
|                               | Splenic cells |                                   |                                   |
|                               | Erythroid    | 0.11                                  | 0.022                             |
|                               | Myeloid      | No expression                         |                                   |
|                               | Lymphoid     | No expression                         |                                   |
| Line F (1)                    | Marrow       | 0.02                                  |                                   |
|                               | Brain        | No expression                         |                                   |
|                               | Heart        | No expression                         |                                   |
|                               | Kidney       | No expression                         |                                   |
|                               | Skeletal muscle | No expression       |                                   |
|                               | Liver        | No expression                         |                                   |
|                               | Lung         | No expression                         |                                   |
|                               | Thymus       | 0.01                                  |                                   |
|                               | Spleen       | 0.04                                  |                                   |
|                               | Splenic cells |                                   |                                   |
|                               | Erythroid    | 0.06                                  | 0.06                              |
|                               | Myeloid      | No expression                         |                                   |
|                               | Lymphoid     | No expression                         |                                   |

FIG. 10. Detection of α-spectrin/λγ-globin mRNA in splenic cells separated into erythroid, myeloid, and lymphoid fractions from transgenic mice containing the α-spectrin/human γ-globin transgene. 1.0 μg of RNA from erythroid, myeloid, or lymphoid cells was hybridized to a 32P-labeled antisense riboprobe which protects exon 2 of the α-spectrin/λγ-globin transgene (top band) and exon 2 of the mouse α-globin gene (lower band), digested with RNase, electrophoresed in an 8% nondenaturing gel, dried, and subjected to autoradiography.

The relative importance of these transcription factor binding sites in promoter function, mutations were introduced into each of the three sites protected in DNase I footprinting experiments and the two upstream GATA-1 sites. Mutation of the upstream GATA-1 consensus sequence (site 1, GATA to GTTA) had no effect on promoter activity (Fig. 4B). Mutating the site 2 GATA-1 consensus sequence in a similar manner (GATA to GTTA) reduced promoter activity by 25%. Mutation of the most 3′ GATA-1 site, site 3, had no effect on promoter activity. Mutation of the NF-E2 site (GCTGAGTCA to TCTGAGTCA) reduced promoter activity by approximately one-half, indicating that this site is of major importance in the α-spectrin gene promoter. When a reporter plasmid with mutations of both the GATA-1 site 2 and NF-E2 sites was transfected into K562 cells, promoter activity was reduced to nearly background (Fig. 4B).

Transgenic Mice Express the α-Spectrin/λγ-Globin Transgene in Erythroid Cells Only at Early Stages of Erythroid Development—We created transgenic mice with a human α-spectrin promoter fragment from −793 to +1 fused to the human λγ-globin gene (Fig. 8A). We also created a dual riboprobe that detects sequences from both exon 2 of the human γ-globin gene and exon 2 of the murine α-globin gene (Fig. 8B). This riboprobe ensures that both human λγ-globin and murine α-globin sequences are labeled to equal specific activity, allowing direct comparison of human λγ-globin and murine α-globin mRNA levels in tissues from transgenic animals. Seven transgenic lines containing the α-spectrin/λγ-globin transgene were analyzed. RNase protection demonstrated that 0 of 7 α-spectrin/λγ-globin transgenic lines expressed the α-spectrin/λγ-globin...
bin transgene in adult reticulocytes (see Table II and Fig. 9). In three lines examined, expression was detected in yolk sac, and lesser amounts were detected in fetal liver (see Table II and Fig. 9). The number of transgenes in each line was estimated by Southern blot analyses to be between 1 and 15 copies per expressing animal. After correction for copy number, the level of α-spectrin/γ-globin mRNA was compared with the mRNA of murine α-globin. Levels of γ-globin expression in yolk sac ranged from 0.04 to 0.08% of the levels of murine α-globin expression in the same cells.

The α-Spectrin/γ-Globin Transgene Does Not Direct Expression in Nonerythroid Tissues—In three transgenic lines examined, the α-spectrin/γ-globin transgene was expressed in adult bone marrow (Table III). In these three lines, the level of transgene expression was examined in nonerythroid tissues of mice who were perfused with saline immediately prior to sacrificing. RNase protection did not detect γ-globin mRNA in kidney, brain, heart, liver, lung, or skeletal muscle mRNA. Low levels of expression were detected in thymus. RNA was isolated from splenic cells harvested from these three transgenic lines and separated into erythroid, myeloid, and lymphoid cells. RNase protection analyses demonstrated low levels of α-spectrin/γ-globin expression only in erythroid cells (see Table III and Fig. 10).

DISCUSSION

Comparison of sequences of the erythroid promoters of ankyrin-1, band 3, and β-spectrin, other erythrocyte membrane proteins, have been performed (44, 46–48). In vitro characterization of the promoters of ankyrin-1, band 3, and β-spectrin, other erythrocyte membrane proteins, have been performed previously (44, 46–48). These studies demonstrated that a combination of GATA-1- and CACCC-binding proteins is essential for high level expression of linked reporter genes. We conclude that the promoters of most erythrocyte membrane protein genes share similarities to other erythroid gene promoters where this combination may lead to cooperation between GATA-1- and CACCC-binding proteins to enhance transcription (49–56). Similarly, consensus binding sites for GATA-1- and CACCC-binding proteins are present in close proximity in the α-spectrin promoter. Our in vitro studies did not demonstrate a role for the CACCC binding site in the α-spectrin gene promoter.

In vitro, the NF-E2 consensus binding sequences in the α-spectrin gene promoter appear to play an important role in promoter function. NF-E2, a heterodimer of two basic leucine zipper proteins of 45 and 18 kDa, plays an important role in erythroid gene expression (57). The 45-kDa proteins, identified as proteins that interact with and activate the NF-E2 site in the β-globin gene locus control region, include p45 NF-E2, the closely related Nr11, Nrf2, Nrf3, and the more distantly related Bach-1 and Bach-2 (58–64). p45 NF-E2 expression is primarily restricted to hematopoietic cells, erythrocytes, neutrophils, megakaryocytes, and mast cells (57). The 18-kDa protein, a member of the small Maf oncoprotein family, is widely expressed (65, 66). NF-E2 proteins have been shown to bind to consensus sequences in the promoters and enhancers of several erythroid and megakaryocytic genes including the β-globin locus control region, porphobilinogen deaminase, ferrochelatase, and thromboxane synthase (37, 67–72). NF-E2-binding proteins have not been shown previously to be involved in expression of other erythrocyte membrane skeleton genes. The core erythrocyte promoters of the ankyrin and β-spectrin genes do not contain NF-E2 consensus binding sequences (44, 46). The erythrocyte band 3 promoter contains consensus binding sequences for NF-E2 (47, 48), but its role in band 3 promoter function has not been examined.

Our studies show that the core promoter of the human α-spectrin gene directs erythroid-specific expression in erythroid cells only in the early stages of erythroid development. This is in contrast to the erythroid promoters of the ankyrin and β-spectrin genes, which direct expression at all stages of erythroid development (44–46). The overall level of expression directed by the core α-spectrin gene promoter was very low, 0.04–0.08% of mouse α-globin/transgene copy number in yolk sac compared with −4 and −3–9% for ankyrin and β-spectrin, respectively (45, 46). This observation was surprising as previous studies had demonstrated that α-spectrin gene expression is quite high in erythroid cells and that the expression was controlled at the transcriptional level (21–23). These data indicate that elements outside the core α-spectrin gene promoter are required for high level expression during erythroid development.

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