A Minimal Ankyrin Promoter Linked to a Human \( \gamma \)-Globin Gene Demonstrates Erythroid Specific Copy Number Dependent Expression with Minimal Position or Enhancer Dependence in Transgenic Mice*

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In red blood cells ankyrin (ANK-1) provides the primary linkage between the erythrocyte membrane skeleton and the plasma membrane. We have previously demonstrated that a 271-bp 5'-flanking region of the ANK-1 gene has promoter activity in erythroid, but not non-erythroid, cell lines. To determine whether the ankyrin promoter could direct erythroid-specific expression in vivo, we analyzed transgenic mice containing the ankyrin promoter fused to the human \( \gamma \)-globin gene. Sixteen of 17 lines expressed the transgene in erythroid cells indicating nearly position-independent expression. We also observed a significant correlation between the level of Ank/\( \gamma \)-globin mRNA and transgene copy number. The level of Ank/\( \gamma \)-mRNA averaged 11% of mouse \( \alpha \)-globin mRNA per gene copy at all developmental stages. The addition of the HS2 enhancer from the \( \beta \)-globin locus control region to the Ank/\( \gamma \)-globin transgene resulted in Ank/\( \gamma \)-globin mRNA expression in embryonic and fetal erythroid cells in six of eight lines but resulted in absent or dramatically reduced levels of Ank/\( \alpha \)-\( \gamma \)-globin mRNA in adult erythroid cells in eight of eight transgenic lines. These data indicate that the minimal ankyrin promoter contains all sequences necessary and sufficient for erythroid-specific, copy number-dependent, position-independent expression of the human \( \gamma \)-globin gene.

The erythrocyte membrane skeleton is composed of a network of structural proteins (\( \alpha \)- and \( \beta \)-spectrin, ankyrin, band 4.1, band 4.2, and actin) linked to integral membrane proteins (band 3 and glycophorin A). Erythrocyte ankyrin (ANK-1) provides the primary linkage between the spectrin/actin oligomers of the erythrocyte membrane skeleton and the cytoplasmic domain of band 3 (AI-1), an integral membrane protein that serves as a major anion exchange protein of the erythrocyte. Through the linkage of the membrane skeleton to the plasma membrane, the erythrocyte is capable of extensive deformability allowing passage through small vessels (1–3).

Besides erythrocytes, ANK-1 mRNA transcripts are found in brain, heart, and skeletal muscle cells (1, 3, 4). In neuronal cells, ANK-1 mRNA initiates at a site distal to the initiation site in erythrocytes (5), whereas a muscle-specific cDNA transcript has been identified that originates between exons 39 and 40 of the ANK-1 gene (6–8). Erythroid ANK-1 transcripts of different sizes have been identified. Two of these transcripts are developmental stage-specific and are generated by alternate use of polyadenylation sites (9, 10). Other ANK-1 transcripts differ in size due to alternative splicing of the primary ankyrin-1 cDNA transcript (5, 10–14).

The ANK-1 erythroid promoter has not been studied in vivo. In our previous in vitro studies we have mapped the 5’ terminus of the ANK-1 mRNA in erythrocyte RNA and characterized the ANK-1 promoter sequences (15). A 271-bp minimal promoter was shown to direct high level expression of a luciferase reporter gene in erythroid cell lines (K562 or HEL) but not in non-erythroid cell lines (HeLa, the neuroblastoma cell line, SH-SY5Y, and the murine myoblast cell line, CCL14). Mutation of a GATA-1 binding site at position –127 abolished luciferase expression in K562 cells while co-transfection of an ANK-1 luciferase reporter gene and a plasmid containing the GATA-1 gene into HeLa cells resulted in transactivation of the reporter gene. Mutation of a CACCC site at position –186 that binds BKLF resulted in a 50% decrease in reporter gene expression (15).

In this study we have used transgenic mice to characterize expression from the ankyrin promoter in erythroid and non-erythroid cells and at different developmental stages. Transgenic mouse lines containing the minimal ANK-1 promoter linked to a human \( \gamma \)-globin reporter gene expressed Ank/\( \gamma \)-globin mRNA in an erythroid-specific, position-independent, copy number-dependent manner in yolk sac, fetal liver, and adult erythroid cells. In transgenic mice that contained the human Ank/\( \gamma \)-globin gene linked to the HS2 site from the \( \beta \)-globin LCR (16), Ank/\( \gamma \)-globin mRNA was expressed in yolk sac and fetal liver-derived erythroid cells at levels similar to that of animals with the Ank/\( \gamma \)-globin transgene, but was absent or expressed at lower levels in adult erythroid cells. We conclude from these data that the minimal ankyrin promoter contains all the sequences necessary and sufficient for erythroid-specific, copy number-dependent expression in vivo.
Fig. 1. Ankyrin/γ-globin transgene constructs. A, the human ANK-1 promoter (−296 to −20) was fused to the human γ-globin gene (−4 to +1902) to create Ank/γ-globin. The ANK-1 promoter is shown in gray. B, a 691-bp fragment containing 5’-HS2 of the human β-globin LCR (GenBank® accession number 455025, coordinates 8486–9177) was fused to Ank/γ-globin to create HS2 Ank/γ-globin. C, the Ank/γ-globin gene riboprobe used as described under “Experimental Procedures.” The riboprobe was transcribed with SP6 polymerase. The probe protects a 169-bp fragment (Ank/γ-globin exon1) and a 175-bp fragment (γ-exon 2).

**Expression of ankyrin/γ-globin mRNA in transgenic mice**

| Cell line | Human γ-globin RNA/mouse a-globin RNA | Transgene copy no. | Mean γ-globin RNA transgene copy no. |
|-----------|--------------------------------------|--------------------|--------------------------------------|
| A         | 0.05 ± 0.01                          | 0.03 ± 0.01        | 0.03 ± 0.01                          | 1 | 0.15 |
| B         | 0.08 ± 0.01                          | 0.06 ± 0.03        | 0.03 ± 0.01                          | 5 | 0.05 |
| H         | 0.06 ± 0.05                          | 0.06 ± 0.01        | 0.06 ± 0.01                          | 4 | 0.06 |
| I         | 0.10 ± 0.10                          | 0.07 ± 0.05        | 0.20 ± 0.05                          | 7 | 0.07 |
| J         | 0.29 ± 0.07                          | 0.20 ± 0.03        | 0.33 ± 0.09                          | 9 | 0.12 |
| K         | 0.62 ± 0.12                          | 0.35 ± 0.17        | 0.47 ± 0.06                          | 26 | 0.07 |
| L         | 0.44 ± 0.19                          | 0.21 ± 0.04        | 0.20 ± 0.01                          | 9 | 0.13 |
| O         | ND                                   | ND                 | ND                                   | 10 | 0.27 |
| D         | ND                                   | ND                 | ND                                   | 4  | 0.16 |
| E         | ND                                   | ND                 | ND                                   | 18 | 0.06 |
| F         | ND                                   | ND                 | ND                                   | 60 | (0.02) |
| G         | ND                                   | ND                 | ND                                   | 17 | 0.00 |
| M         | ND                                   | ND                 | ND                                   | 15 | 0.06 |
| N         | ND                                   | ND                 | ND                                   | 32 | 0.06 |
| O         | ND                                   | ND                 | ND                                   | 22 | 0.06 |
| Q         | ND                                   | ND                 | ND                                   | 8  | 0.28 |

Mean 0.21 ± 0.22 0.14 ± 0.11 0.25 ± 0.20 0.11 ± 0.08

**Experimental Procedures**

**Plasmid Constructs**—To generate Ank/γ-globin, a 276-bp Smal/BglII fragment containing the ANK-1 promoter (−291 to −20 + polylinker sequence) was excised from a pGL2 vector (15). A 1938-bp BglII/HindIII γ-globin fragment from plasmid 72bp + γ (described in Sabatino et al.) (17). A triple ligation consisting of both fragments and SmaI/HindIII-digested pSP72 was used to generate the Ank/γ-globin plasmid. The Ank/γ-globin gene (2244 bp) was excised from the plasmid with EcoRV and HindIII for microinjection.

HS2 Ank/γ-globin was generated by a four-part ligation using a 712-bp Xhol/HindIII fragment containing HS2 (GenBank® accession number 455025, coordinates 8486–9177 + polylinker sequence), the 1938-bp BglII/HindIII fragment containing the γ-globin gene, the 276-bp Smal/BglII fragment containing the ankyrin promoter, and the vector pBluescript II KS + cut with Xhol/HindIII. The 2917-bp HS2 Ank/γ-globin gene was excised from this plasmid with ScaI and HindIII for microinjection. Both constructs were sequenced to confirm that the ANK-1 promoter was correctly fused to the γ-globin gene.

**Generation of Transgenic Mice**—Transgenic mice were generated as described in Hogan et al. (18). Fertilized eggs were collected from superovulated FVB/N female mice (Taconic Farms) approximately 9 h after mating to CByB6F1 male mice (Jackson Laboratory). Fragments for microinjection were separated on an agarose gel, electroeluted, and purified with an Elutip-d minicolumn (Schleicher and Schuell, Inc.). The fragments were diluted to a concentration of 2 mg/ml in 10 mM Tris, 0.25 mM EDTA (pH 7.5), and −2 µ1 was injected into the male pronucleus of fertilized eggs. The injected eggs were transferred to pseudo-pregnant CByB6F1 foster mothers. Founder animals were identified by Southern analysis of DNA extracted from tail biopsies by probing with an Ank/γ-globin probe. Founder animals were crossed to FVB/N mice for propagation and developmental studies.

**Transgene Copy Number Analysis**—Copy number was determined by comparing the γ-globin signals from Southern blot analysis of transgenic mouse and K562 DNA (19) using a Molecular Dynamics PhosphorImager. Statistical analysis of copy number and expression data was analyzed by linear regression with the Graph Pad Prism Version 2.0 software.

**Isolation of RNA**—Reticulocytes were obtained by collecting 200 µl of blood from phlebotomized animals. Tissues were collected from anesthetized animals perfused with 50–60 ml of saline, and the tissues were stored in liquid nitrogen. Total cellular RNA was extracted from adult reticulocytes, 10.5 dpc of embryo blood cells, 13.5 dpc of fetal livers, and tissues using Trizol reagent according to the manufacturer’s specifications (Life Technologies, Inc.).

**RNase Protection Assays**—Linear DNA templates for the RNase protection assay were prepared by EcoRI (Ank/γ) and HindIII (mouse a) digestion of cesium chloride-purified plasmid preparations. The templates were purified by agarose gel electrophoresis and purified using a GeneClean II kit (Bio 101, Inc.). The linear DNA template for the mouse β-actin gene was obtained from the MAXScript in vitro transcription kit (Ambion, Inc.). 32P-Labeled RNA probes were transcribed using the same kit. The hybridization of the probe and RNA (1–10 µg) was carried out overnight according to the standard procedure of the RPA II of the RNase protection assay kit (Ambion, Inc.). RNase digestion was performed using an RNase A/RNase T1 mixture in RNase digestion buffer (Ambion, Inc.), and the protected fragments were separated on an 8% denaturing polyacrylamide gel.

**Quantitation of mRNA Levels**—To quantitate the levels of mRNA, the gel was exposed to a PhosphorImager screen and scanned on a Molecular Dynamics PhosphorImager. The relative amounts of the...
Ankyrin Promoter Function in Transgenic Mice

Fig. 2. Detection of Ankαγ-globin mRNA in reticulocytes of transgenic mice. 1 μg of RNA from adult reticulocytes was hybridized to 32P-labeled antisense RNA probes for the Ankαγ-globin transgene (top panel) and mouse α-globin (lower panel) and digested with RNase. The positions of protected fragments corresponding to 14 γ-globin exon 2 (175 bp), properly initiated Ankαγ-globin exon 1 (169 bp), and mouse α-globin (186 bp) are indicated. 10 μg of RNA from K562 cells was analyzed as a positive control (extreme left lane). The letters above the lanes indicate the individual strains of transgenic mice (14 of 17 cell lines are shown, including the only negative line).

RESULTS

Transgenic Mice with Human Ankyrin 1γ-globin Gene Constructs—The 271-bp minimal ANK-1 promoter fragment was fused to the human 1γ-globin genomic sequence immediately upstream of the ATG initiation codon (Ank1αγ-globin) (Fig. 1A). A second construct added 5′-HS2 of the human β-globin LCR gene immediately 5′ of Ank1αγ-globin (HS2 Ank1αγ-globin) (Fig. 1B). Seventeen transgenic mouse lines were generated with the Ank1αγ construct. Southern blot analysis determined that the transgene copy number of these lines ranged between 1 and 100 (Table I). RNase protection analysis demonstrated that 16 of these 17 lines expressed Ank1αγ-globin mRNA in adult reticulocytes (Fig. 2). The probe protects both exon 2 of the γ-globin gene and the chimeric exon 1 of the Ank1αγ-globin gene. The size of the upper band corresponds to the predicted size for properly spliced γ-globin exon 2 (Fig. 2). The size of the lower band corresponds to the predicted size of properly spliced Ank1αγ-globin exon 1 initiated at a site described previously for ANK-1 mRNA in K562 cells (15).

The ratio of Ank1αγ-globin mRNA to mouse α-globin mRNA was compared with the transgene copy number in the 16 lines that expressed Ank1αγ-globin mRNA. An analysis of the nine lines with a copy number of ≤10 demonstrated a significant linear correlation between copy number and γ-globin expression (p < 0.05; Fig. 3, top panel). An analysis of all 16 lines that expressed the γ-globin gene demonstrated a significant exponential correlation between the copy number and transgene expression (p < 0.01; Fig. 3, bottom panel). The average level of Ank1αγ mRNA in the 16 expressing Ank1αγ-globin transgenic mouse lines was 11% (±8%) that of the mouse α-globin per transgene copy (Table I).

Distribution of Ankyrin 1γ-globin mRNA in Non-erythroid Tissues of Transgenic Mice—RNase protection analysis of Ank1γ-globin mRNA levels was performed on RNA extracted from 10 different tissues from perfused mice of four different Ank1γ-globin lines. The Ank1α-globin transgene was expressed in reticulocytes (Fig. 2), bone marrow, and spleen cells (Fig. 4; Table II). Occasional Ank1α-globin mRNA transcripts were detected at low levels in other tissues. However, this mRNA was always present along with mouse α-globin mRNA, and the ratio of Ank1α-globin mRNA to mouse α-globin mRNA was nearly the same in the tissues as the ratio in bone marrow, spleen, and reticulocyte RNA (data not shown). We conclude that these signals represent low level contamination of the tissue RNA with reticulocytes, but we cannot completely exclude low levels of expression.

Expression of Ank1α-globin mRNA during Mouse Development—RNA was collected from 10.5 dpc of yolk sac-derived peripheral blood cells, 13.5 dpc of fetal livers, and adult reticulocytes from seven of the Ank1α-globin transgenic lines. RNase protection analysis demonstrated that Ank1αγ was expressed at similar levels in yolk sac, fetal liver, and adult erythroid cells in all seven lines analyzed. The average levels of Ank1αγ mRNA during development ranged between 5% and 15% that of the endogenous mouse α-globin per transgene copy (Fig. 5; Table I).
ber for the transgenic lines ranged between 1 and 39 (Table III). RNA was isolated from 10.5 dpc of yolk sac-derived erythroid cells, 13.5 dpc of fetal liver-derived erythroid cells, and adult reticulocytes of all eight lines. RNase protection analysis demonstrated that five transgenic lines had Ank^Aγ-globin mRNA present at an average level of 18 ± 16% of the level of mouse α-globin mRNA in yolk sac–derived erythroid cells, 9 ± 8% of mouse α-globin mRNA in fetal liver–derived erythroid cells, and no Ank^Aγ mRNA in the adult reticulocytes (Fig. 6; Table III). One transgenic line expressed Ank^Aγ-globin mRNA at all stages of development, however, the level of expression in yolk sac erythroid cells was 22% that of mouse α-globin in the yolk sac and decreased to 4% that of the mouse α-globin in adult erythroid cells (Fig. 6; Table III). In contrast to the Ank^Aγ-globin transgenic mice where 16 of 17 lines expressed the transgene, two HS2 Ank^Aγ-globin transgenic mouse lines did not express Ank^Aγ mRNA at any developmental stage (χ² = 4.8; p < 0.05) (Fig. 6; Table III).

**DISCUSSION**

Our data are consistent with the hypothesis that the 271-bp minimal ANK-1 promoter contains all of the sequences necessary and sufficient for position-independent, copy-number-dependent expression of the Ank^Aγ-globin gene. The significant exponential correlation between transgene copy number and mRNA level in all 16 lines is consistent with a model in which erythroid transcription complexes become limiting when the copy number exceeds 10 copies. In this model additional trans-gene copies would generate incremental rather than linear increases in the level of Ank^Aγ-globin mRNA. It is less likely that the level of Ank^Aγ-globin mRNA is controlled by the rate of RNA degradation, because globin mRNA is stable in erythroid cells (20).

The effect of the 5′-HS2 on expression of the Ank^Aγ-globin gene differs markedly from the effects of 5′-HS2 on the human ß-, γ-, and β-globin genes in other transgenic mouse models. In the absence of HS2, globin genes are expressed at low levels (<1% of the endogenous mouse gene) in 50% or less of transgenic lines (21–25), and show either yolk sac (γ)- or fetal liver/ adult (β)-specific expression. The presence of 5′-HS2 relieved the position dependence of linked globin genes and increased the level of expression 10-fold or more, but the genes were expressed at all stages of development (26–29). Previously, we have showed that the ß-spectrin promoter can also be influenced by the 5′-HS2 (17). Expression of a ßsp/Aγ-globin transgene was position-dependent, because only three of five lines expressed the ßsp/Aγ mRNA in erythroid cells. The addition of 5′-HS2 to the ß-spectrin promoter relieved the position dependence (seven of seven lines expressed) but had no effect on the level of expression (17). In these studies 5′-HS2 caused the Ank^Aγ-globin transgene to be expressed in a position-dependent, stage-specific pattern. We conclude that, unlike the globin or ß-spectrin promoters, the ANK-1 promoter is not sensitive to the enhancer effects of 5′-HS2. Further dissection will be required to determine the basis of these observations.

The sequence of the ankyrin promoter differs from many other erythroid-specific gene promoters. Similar to housekeeping promoters, the ANK-1 promoter is GC-rich and does not contain consensus TATA, InR, or CCAAT sequences. In contrast, globin gene promoters contain both TATA and CCAAT elements, stage-specific pattern. We conclude that, unlike the globin or ß-spectrin promoters, the ANK-1 promoter is not sensitive to the enhancer effects of 5′-HS2. Further dissection will be required to determine the basis of these observations.

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**Table II**

| Tissue | Line L | Line O | Line K | % Ank^Aγ-mRNA^a |
|--------|--------|--------|--------|-----------------|
| Marrow | 100.00 | 100.00 | 100.00 |
| Spleen | 2.30   | 2.30   | 7.80   |
| Thymus | 0.03   | 1.00   | 0.24   |
| Brain  | 0.01   | 1.60   | 0.01   |
| Heart  | 0.33   | 3.10   | 0.16   |
| Kidney | 0.51   | 0.32   | 0.01   |
| Liver  | 0.27   | 0.00   | 0.01   |
| Lung   | 0.01   | 0.00   | 0.02   |
| Muscle | 3.60   | 0.52   | 0.18   |
| Testis | 1.06   | 0.06   | 0.01   |

^a^ Ank^Aγ-globin mRNA in tissue/Ank^Aγ-globin mRNA in marrow × 100.

**Fig. 4.** Analysis of Ank^Aγ-globin mRNA levels in transgenic mouse tissues. A transgenic mouse from cell line L was perfused with 50–60 ml of saline prior to dissection and RNA collection. For the RNase protection analysis, the RNA was hybridized to antisense RNA probes for the human Ank^Aγ-globin (top panel), mouse α-globin (middle panel), and mouse β-actin genes (bottom panel). The positions of protected fragments corresponding to Ank^Aγ-globin, mouse α-globin, and mouse β-actin are indicated. The panels shown were not exposed for the same length of time.

**Fig. 5.** Analysis of Ank^Aγ-globin mRNA levels in yolk sac, fetal liver, and adult erythroid cells of transgenic mice containing the Ank^Aγ-globin transgene. RNA was isolated from yolk sac–derived erythroid cells, fetal liver–derived erythroid cells, and adult reticulocytes. RNase protection analysis of the RNA hybridized to antisense RNA probes corresponding to the Ank^Aγ-globin (upper panels) and mouse α-globin (lower panels) genes. Three transgenic mouse lines are shown (cell lines H, K, and L) and are representative of the seven lines that were analyzed.
for erythroid-specific, position-independent expression of the γ-globin transgene. In contrast, the β-globin locus CCR is a cis-regulatory element located 10–60 kb upstream of the β-like globin genes and is required for their high level position-independent expression (16, 32, 33). The promoter of the hematopoietic-specific transcription factor, GATA-1, which is important for expression of genes of erythroid and other hematopoietic cell lineages (34), is similar to the ANK-1 promoter, because it lacks TATA and CCAAT sequences (35, 36) but requires a DNase I-hypersensitive site located 3.7 kb upstream of the transcription start site for high level erythroid-specific expression (37). Finally, the porphobilinogen deaminase gene requires a region that is DNase I-hypersensitive located 1 kb upstream of the transcription start site for position-independent, erythroid-specific expression (38).

The minimal ANK-1 promoter contains a GATA-1 binding site at position −128 (15, 34) and a basic Kruppel-like factor binding site at −186 (15). Both of these sites are important for trans activation of a luciferase reporter gene in transient assays (15). Ankyrin deficiency has been shown to be the most common cause of hereditary spherocytosis (39). Eber et al. (40) described mutations at positions −204 (C to G) and −108 (T to C) in the ANK-1 promoter, which they postulated caused a decreased synthesis of ANK-1 mRNA. Neither of these variants involve the GATA-1 or basic Kruppel-like factor binding sites, suggesting that if the −204 and −108 variations are disease-causing mutations, additional binding proteins may be involved in ANK-1 gene erythroid transcription.

One of our interests is to evaluate the expression of the Ankyrin/γ-globin gene delivered to erythroid cells by a retroviral vector as a potential gene therapy of the hemoglobinopathies. The development of globin retrovirus vectors has been hampered by unstable gene transfer and position-dependent, variated expression of globin genes with LCR elements. The high level, erythroid-specific, position-independent expression of the Ankyrin/γ-globin gene makes it a good candidate for a retroviral vector. If the Ankyrin/γ gene can be expressed in the same fashion in a retroviral vector, it could provide therapeutic levels of γ-globin in erythroid cells of patients with sickle cell disease and β-thalassemia.

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A Minimal Ankyrin Promoter Linked to a Human γ-Globin Gene Demonstrates Erythroid Specific Copy Number Dependent Expression with Minimal Position or Enhancer Dependence in Transgenic Mice

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