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A dinucleotide deletion in the ankyrin promoter alters gene expression, transcription initiation and TFIID complex formation in hereditary spherocytosis

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Ankyrin defects are the most common cause of hereditary spherocytosis (HS). In some HS patients, mutations in the ankyrin promoter have been hypothesized to lead to decreased ankyrin mRNA synthesis. The ankyrin erythroid promoter is a member of the most common class of mammalian promoters which lack conserved TATA, initiator or other promoter *cis* elements and have high G+C content, functional Sp1 binding sites and multiple transcription initiation sites. We identified a novel ankyrin gene promoter mutation, a TG deletion adjacent to a transcription initiation site, in a patient with ankyrin-linked HS and analyzed its effects on ankyrin expression. *In vitro*, the mutant promoter directed decreased levels of gene expression, altered transcription initiation site utilization and exhibited defective binding of TATA-binding protein (TBP) and TFIID complex formation. In a transgenic mouse model, the mutant ankyrin promoter led to abnormalities in gene expression, including decreased expression of a reporter gene and altered transcription initiation site utilization. These data indicate that the mutation alters ankyrin gene transcription and contributes to the HS phenotype by decreasing ankyrin gene synthesis via disruption of TFIID complex interactions with the ankyrin core promoter. These studies support the model that in promoters that lack conserved *cis* elements, the TFIID complex directs preinitiation complex formation at specific sites in core promoter DNA and provide the first evidence that disruption of TBP binding and TFIID complex formation in this type of promoter leads to alterations in start site utilization, decreased gene expression and a disease phenotype *in vivo*.

INTRODUCTION

Hereditary spherocytosis (HS) is a common inherited hemolytic anemia that affects all ethnic groups. The primary cellular abnormality in HS is loss of erythrocyte membrane surface area relative to intracellular volume, leading to spherical shaped erythrocytes with decreased deformability. Defects of the erythrocyte membrane protein ankyrin (ankyrin-1, ANK1, OMIM 182900) are the most common cause of HS (1–4). In some ankyrin-deficient HS patients, sequence variations in the ankyrin core promoter region have been hypothesized to lead to decreased ankyrin mRNA synthesis (1,5,6). It is not known whether these are disease-causing mutations or merely polymorphisms.

Significant progress has been made in our understanding of core promoter structure and function. Core promoters have been best studied in model organisms, particularly *Drosophila*, which usually contain combinations of conserved *cis* elements including a TATA box, a transcription factor IIB recognition element, an initiator element (InR), a downstream promoter

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element (DPE) or the motif ten element (7–16). Mammalian core promoters are more complex and may contain a TATA box alone, a TATA box and an InR, an InR and a DPE etc. or contain none of these conserved *cis* elements (8,17).

The ankyrin erythroid promoter is a member of the most common class of mammalian promoters (>50%) (18) which lack conserved *cis* elements and have high C+G content, functional upstream Sp1 binding sites and multiple transcription initiation sites. In this type of promoter, Sp1 activation directs the general transcription machinery to form preinitiation complexes in a region ~40–100 bp downstream of its binding sites (19,20). In the downstream region, it has been hypothesized that the TATA-binding protein (TBP) of the TFIID complex directs preinitiation complex formation at the sequences in the promoter DNA that most closely resemble TATA or InR elements (17). Disease-associated mutations in mammalian promoters lacking conserved *cis* elements have not been well characterized. Mutation of known DNA-transcription factor binding sites or conserved core promoter *cis* sequences provides understandable causes for alterations in gene expression, but in most cases, the functional significance of sequence variations in the promoter region is unknown (21–26).

We have identified a novel erythroid ankyrin gene core promoter mutation, a TG deletion dinucleotide at position −72/−73 adjacent to a transcription initiation site, in a patient with ankyrin-linked HS. *In vitro* and *in vivo* studies demonstrated that the mutant promoter directed decreased gene expression and led to alterations in transcription initiation site utilization. The mutant ankyrin promoter also demonstrated defective TBP binding and TFIID complex formation. We conclude that the −72/−73 mutation alters ankyrin gene transcription and contributes to the HS phenotype by decreasing ankyrin synthesis via disruption of TFIID complex interactions with the ankyrin core promoter. These studies support the model that in promoters lacking conserved *cis* elements, the TFIID complex directs preinitiation complex formation at sites in core mammalian promoter DNA that functionally resemble TATA or InR elements (17). Similar sequence variants identified in other genes with this type of promoter have been associated with a disease phenotype, suggesting that disruption of TFIID complex formation in this type of promoter is an under-recognized cause of genetic disease.

RESULTS

Qualitative and quantitative analyses of erythrocyte membrane proteins

One-dimensional SDS–PAGE analyses of erythrocyte membranes from the proband and her mother were qualitatively normal, and no abnormal isoforms of any major membrane proteins were observed (data not shown). Quantitative analyses of spectrin and ankyrin content, as measured by the ratios of spectrin/band 3 and ankyrin/band 3, respectively, are shown in Table 1. The erythrocyte membranes of mother demonstrate combined ankyrin and spectrin deficiency at a level typical for other cases of ankyrin-linked, mild-to-moderate HS, ~70–90% (1). Combined ankyrin–spectrin deficiency is a common finding in ankyrin-deficient erythrocytes, as decreased or defective ankyrin membrane assembly may lead to decreased availability of spectrin-binding sites on ankyrin (2,27). Splenectomy is curative in most patients, even those with severe HS and membrane protein deficiency, as splenic sequestration of abnormal erythrocytes is the primary determinant of erythrocyte survival in HS patients (2,3). As is typical postsplenectomy, the membranes of proband exhibited only mild-to-moderate ankyrin deficiency because splenectomy reduces hemolysis, prolongs erythrocyte life span, decreases the reticulocyte count and normalizes erythrocyte membrane protein quantitation (27). In addition, her membranes demonstrated a decreased ratio of protein 4.1a to protein 4.1b, indicating a young population of erythrocytes consistent with her postsplenectomy compensated hemolysis. Prior to splenectomy, the proband suffered from moderate to severe hemolytic anemia, Hb 8–10 g/dl, with reticulocytosis and hyperbilirubinemia. This necessitated splenectomy in childhood. The parents both suffered from mild HS with compensated hemolysis. Neither parent required splenectomy.

SSCP analyses and nucleotide sequencing

Screening of the coding region exons of the ankyrin gene previously identified a 20 bp deletion in exon 6 of one allele from the proband (1). This deletion leads to frameshift and premature chain termination, creating effectively a null allele. The deletion was confirmed by single-stranded conformational polymorphism (SSCP) analyses and nucleotide sequencing of exon 6 amplification products from the proband. The deletion was not observed in exon 6 amplification products from her mother (data not shown). The father is presumed to have the 20 bp exon 6 deletion as the cause of his HS phenotype. However, he is deceased and genetic material to confirm this is not available.

To search for additional HS mutations, the ankyrin gene promoter region was screened by SSCP of amplified genomic DNA from the proband and her ankyrin-deficient mother. An abnormal SSCP pattern was identified in the promoter region in two separate PCR/SSCP reactions in the proband and her mother (data not shown). Analysis of the corresponding DNA sequence revealed a TG dinucleotide deletion at position −72/−73 relative to the ATG initiation codon (Fig. 1). The DNA sequence at and around −72/−73 does not contain consensus sequences for known DNA binding proteins, but −72/−73 is adjacent to one of the ankyrin transcription initiation sites.

Transfection assays

Plasmids containing wild-type or mutant −72/−73 ankyrin promoter fragments linked to a firefly luciferase reporter gene were transiently transfected into HeLa and K562 cells. The relative luciferase activity was determined 24 h after transfection and compared to the activity obtained with a promoterless-luciferase negative control and a SV40 early promoter-luciferase positive control. The −72/−73 mutant ankyrin promoter directed statistically lower levels of luciferase reporter gene expression than the wild-type promoter in both cell types (Table 2).

Table 1. Clinical, biochemical and molecular studies

	Hb (g/dl)	Reticulocyte count (%)	Osmotic fragility	Splenectomy	(% of normal)		Mutation
					Ankyrin	Spectrin	
I-1 (mother)	13.5	8	Abnl	No	83.3	82.5	Promoter −72/−73 TG
I-2 (father)	17.0	8	Abnl	No	Unknown[a]	Unknown[a]	
II-1 (proband)	12.3	14	Abnl	Yes	89[b]	103	1: Exon 6, 20 bp deletion; 2: Promoter, −72/−73 TG

[a]The father is deceased and genetic material is not available.
[b]Values are postsplenectomy.

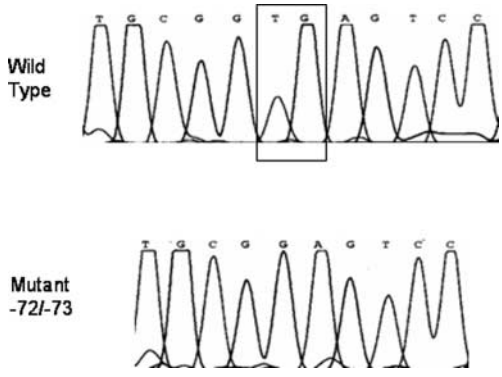

Figure 1. Nucleotide sequence of subcloned PCR products corresponding to the ankyrin promoter from the proband. Sequence of the wild-type allele. The TG dinucleotide deleted in the mutant is boxed (top). Sequence of the mutant allele with the TG deletion at −72/−73 (bottom).

Table 2. Transient transfection analyses

Promoter/reporter	Cell type	
	K562	HeLa
Wild-type ankyrin promoter/luciferase	45.5 ± 7.2	21.3 ± 1.7
Mutant −72/−73 ankyrin promoter/luciferase	38.0 ± 6.0[a]	9.4 ± 0.6[b]
SV40 promoter/luciferase	59.2 ± 3.3	74.6 ± 0.7
Promoterless/luciferase	1	1

Data presented are mean ± SD.
[a]$n = 12$, $P < 0.05$.
[b]$n = 9$, $P < 0.0005$, both Student's *t*-test.

In vitro transcription assays

In a cell-free *in vitro* transcription system using a run-off technique, transcription from wild-type and mutant promoter templates was analyzed with K562 cell nuclear extracts. When analyzed in a quantitative slot blot assay, total transcription products generated by the mutant promoter were decreased to 53.2 ± 7.6% ($n = 3$, $P = 0.012$) in K562 cell extracts when compared with wild-type (Fig. 2, A).

Run-off products generated with HeLa cell extracts were analyzed by acrylamide gel electrophoresis. HeLa cells are enriched in basal transcription factors and are considered by many to be the model system of choice for detailed study of mammalian core promoters. The wild-type promoter template generated bands corresponding to six transcription initiation sites, identical to that seen in human erythroid cells (18). In run-off transcription products generated by the mutant −72/−73 promoter, the band corresponding to initiation site 4, immediately adjacent to the TG deletion, was not observed (Fig. 2, B), and the intensity of the upstream start sites, particularly site 1, was increased.

Transgenic mice with human ankyrin promoter/$^{A}\gamma$-globin reporter genes

We have previously demonstrated that ankyrin promoter mutations can lead to defects in expression of a linked γ-globin reporter gene in transgenic mice (28). We utilized this model system to study the −72/−73 ankyrin promoter mutation. Wild-type or −72/−73 mutant ankyrin promoter fragments were fused to the human $^{A}\gamma$-globin sequence immediately upstream of the ATG initiation codon. Sixteen wild-type and eight −72/−73 mutant transgenic mouse lines were analyzed. Southern blot analysis determined that the transgene copy number in these mice ranged between 1 and 15 (Table 3).

RNase protection analysis of ankyrin/$^{A}\gamma$-globin transgene and endogenous murine α-globin gene expression was performed with reticulocyte RNA using a riboprobe that contains human $^{A}\gamma$-globin and murine α-globin gene sequences on the same transcript, allowing direct comparison of human $^{A}\gamma$-globin and murine α-globin mRNA levels. All 16 lines with the wild-type ankyrin promoter expressed the $^{A}\gamma$-globin transgene (Table 4), and there was a significant correlation with copy number ($r^2 = 0.59$, $P < 0.001$; Fig. 3, top, and Table 4) (29). $^{A}\gamma$-globin mRNA levels were significantly decreased in transgenic mice with the −72/−73 mutant ankryin promoter when compared with transgenic mice with the wild-type ankyrin promoter ($P < 0.02$) (Fig. 3, bottom, and Table 4). Similar to wild-type promoter mice, $^{A}\gamma$-globin mRNA levels from mutant −72/−73 promoter mice correlated with copy number ($r^2 = 0.846$, $P < 0.002$). We have previously shown that transgenic mice with the wild-type ankyrin promoter express human $^{A}\gamma$-globin in a uniform pattern, i.e. in 100% of erythrocytes (28). We have also shown that other sequence variants in the ankyrin promoter region, −108 T–C and −153 G–A, lead to non-uniform, varigated expression in transgenic mice (28). Therefore, we analyzed the expression pattern of human $^{A}\gamma$-globin protein in the red cells of mutant −72/−73 transgenic mice. Similar

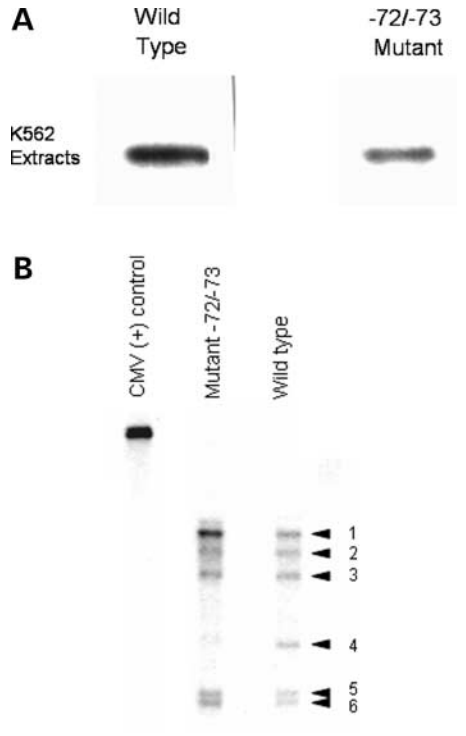

Figure 2. *In vitro* transcription. (**A**) A cell-free *in vitro* transcription system using a run-off technique was used to analyze transcription from wild-type and mutant promoter templates. In a quantitative slot blot assay with K562 cell extracts, transcription products generated by the mutant promoter were decreased to 53.2 ± 7.6% ($P = 0.012$) compared with wild-type (**B**). Analysis of run-off transcription products generated with HeLa cell extracts by gel electrophoresis demonstrated that the transcription initiation site immediately adjacent to the TG deletion, site 4, was absent in transcription products generated by the mutant promoter when compared with the wild-type promoter (right).

to wild-type, the mutant promoter expressing transgenic lines demonstrated a uniform pattern of $^{A}\gamma$-globin protein expression (Table 4). We conclude that the mutations at −108 and −153 are distinct from the effects of the −72/−73 mutation.

We analyzed $^{A}\gamma$-globin mRNA expression in reticulocyte RNA to determine whether transcription was properly initiated in transgenic mice with the mutant ankyrin promoter. Hybridization of a wild-type riboprobe to RNA from wild-type promoter transgenic mice (Fig. 4, left, WT) revealed four RNA transcription start sites corresponding to sites 1, 2, 3 and 4, previously identified in erythroid cell RNA (18) and in our *in vitro* transcription studies. The two smallest bands, corresponding to sites 5 and 6, were not found in either wild-type or mutant −72/−73 transgenic mice. After hybridization of the labeled wild-type riboprobe to RNA from mutant −72/−73 transgenic mice, RNase digestion at the site of the dinucleotide deletion produced only a single protected fragment truncated at position −72, demonstrating the presence of the −72/−73 mutation in these animals (Fig. 4, left, −72/−73).

To map the transcription initiation sites in −72/−73 promoter transgenic mice, a mutant −72/−73 ankyrin promoter riboprobe was used. When this probe was hybridized to RNA from wild-type promoter transgenic mice, four protected fragments were generated. These fragments were identical in their frequency to those protected by the wild-type probe, but were two nucleotides smaller (Fig. 4, right). When this riboprobe was hybridized to RNA from transgenic mice expressing the −72/−73 promoter transgene, the band corresponding to RNA initiation at site 4 was not observed, the relative amount of RNA initiated at site 3 was significantly decreased (wild-type 48 ± 0.2%, $n = 3$ versus mutant 23.7 ± 1%, $n = 5$, $P < 0.001$) and the relative amounts of RNA initiated at site 1 (wild-type 16 ± 2% versus mutant 40.1 ± 1%, $P < 0.001$) and site 2 (20.6 ± 4% versus 36.3 ± 1%, $P < 0.02$) were significantly increased (Fig. 4, left). The absence of site 4 and increased usage of upstream sites 1 and 2 are consistent with the results from *in vitro* transcription studies. We conclude that the −72/−73 deletion alters both the number of transcription initiation sites and the frequency with which they are used in transgenic mice.

In vitro analyses of DNA–protein interactions

Oligonucleotide probes (Table 2) corresponding to the wild-type and mutant ankyrin promoter sequences were analyzed in standard acrylamide gel electrophoretic mobility shift analyses (EMSAs) with K562 cell nuclear extracts. The wild-type probe formed a slow migrating, very high molecular weight complex not seen when using the mutant −72/−73 probe (data not shown). The composition of this high molecular weight complex was unknown, but we hypothesized that it involved a TFIID-containing complex because −72/−73 is immediately adjacent to a transcription initiation site and, in core promoters lacking conserved *cis* elements like the ankyrin promoter, Sp1 directs preinitiation complex formation downstream of its binding sites (7), likely via TFIID complexes directing preinitiation complex formation at sites in the DNA that resemble TATA or InR elements (7).

To study TFIID complex formation, we utilized the well-characterized magnesium agarose gel EMSA system with combinations of recombinant TFIIA, TFIIB and TBP to study ankyrin promoter–TFIID complex formation. This system specifically overcomes the limitations of standard acrylamide EMSA conditions used in our initial studies, such as poor mobility of TFIID complexes in acrylamide gels and epitope inaccessibility preventing detailed analyses in competition and super-shift assays. Wild-type and mutant −72/−72 ^{32}P-labeled ankyrin gene promoter fragments from −296 to −15 were used as probes. Complex formation was most robust with the wild-type probe when a combination of recombinant TFIIA and recombinant TBP were used. The mutant probe exhibited reduced binding under the same conditions, 39.9 ± 4.7% ($n = 5$, $P = 0.0002$) of wild-type control (Fig. 5A).

These data indicated that TFIID complexes are able to form at the ankyrin promoter. To determine whether complexes formed around the region of the −72/−73 mutation, we assessed binding of TBP, the protein of the TFIID complex that contacts DNA, to the −72/−73 promoter region by *in vitro* DNase I footprinting. Wild-type and mutant ankyrin promoter probes were incubated with recombinant TBP, recombinant TBP plus recombinant TFIIA or HeLa cell nuclear extracts. After digestion and gel electrophoresis, all

Table 3. Expression of $^{A}\gamma$-globin in transgenic mice

Transgenic line	Transgene copy number	γ-globin mRNA/murine α-globin mRNA	γ-globin mRNA/murine α-globin mRNA per copy	Percent γ-globin protein positive erythrocytes
Wild-type				
A	1	0.020	0.02	100
B	1	0.023	0.023	ND
C	2	0.112	0.056	100
D	1	0.039	0.039	100
E	2	0.055	0.028	ND
F	15	0.400	0.027	ND
H	1	0.047	0.047	100
I	2	0.123	0.062	ND
J	3	0.169	0.056	100
K	6	0.281	0.047	ND
L	2	0.128	0.064	ND
M	3	0.129	0.043	ND
N	5	0.198	0.040	ND
O	4	0.244	0.061	ND
P	3	0.111	0.037	ND
Q	12	0.140	0.017	100
		Correlation: $r^2 = 0.59$, $P < 0.001$	Mean ± SD: 0.044 ± 0.014[a]	
Mutant −72/−73				
A	11	0.479	0.044	100
B	1	0.026	0.026	ND
C	13	0.344	0.026	100
D	1	0.002[b]	0.002	ND
E	3	0.006[b]	0.002	ND
F	5	0.132	0.026	100
G	2	0.033	0.017	100
H	4	0.192	0.048	100
		Correlation: $r^2 = 0.846$, $P < 0.002$	Mean ± SD: 0.024 ± 0.016[c]	

[a] $P < 0.02$ compared with −72/−73.
[b] Values ∼0.008 and lower are indistinguishable from background.
[c] $P < 0.02$ compared with wild-type.

Table 4. Oligonucleotide primers

SSCP primers	
Promoter A	
Sense	5′-CAGGGCCGAAGCTTCCTCTAC-3′
Antisense	5′-CGGGGAGAGCTGAGTTCAGAG-3′
Promoter B	
Sense	5′-GCGACTAAACCGGACTCCCTTTC-3′
Antisense	5′-AGGAGGAGCAGCTGGGGC-3′
Exon 6	
Sense	5′-GCTGGCGTCAGACGAGTCAGA-3′
Antisense	5′-AGCTCCTCCCTCCTCCTCGC-3′
Electrophoretic mobility shift primers	
Wild-type	
Sense	5′-GCAGAGGCTGCGGTGAGTCCGCCAGCC-3′
Antisense	5′-GGCTGGCGGACTCACCGCAGCCTCTGC-3′
Mutant −72/−73	
Sense	5′-GCAGAGGCTGCGGAGTCCGCCAGCC-3′
Antisense	5′-GGCTGGCGGACTCCGCAGCCTCTGC-3′

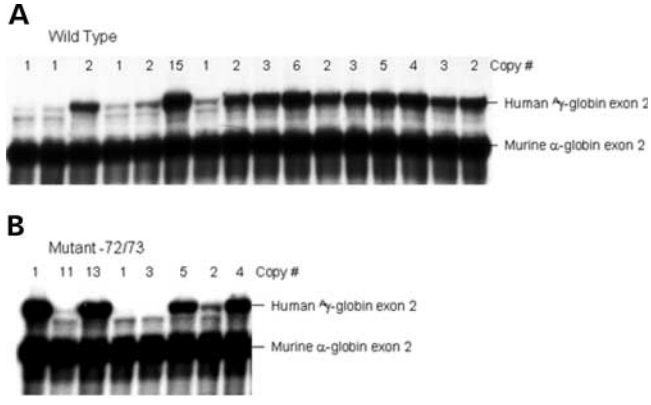

Figure 3. Detection of ankyrin/$^{32}\gamma$-globin mRNA in reticulocytes of transgenic mice. An aliquot of 0.1 μg of RNA from adult reticulocytes was hybridized to the ^{32}P-labeled antisense riboprobe that protects exon 2 of the ankyrin/$^{A}\gamma$-globin transgene (top band) and exon 2 of the mouse α-globin gene (lower band) and digested with RNase. (**A**) Wild-type ankyrin promoter transgenic mice. (**B**) Mutant −72/−73 ankyrin promoter transgenic mice. The numbers above each lane indicate transgene copy number. Samples from individual strains are shown from left to right in alphabetical order (Table 4).

three combinations of proteins demonstrated a protected region from −68 to −75 with a wild-type ankyrin promoter probe. Consistent with the magnesium agarose EMSA results, this region was not protected when the mutant −72/−73 ankyrin promoter probe was used (Fig. 5B).

DISCUSSION

Variability in the clinical severity in HS kindreds has been well described, but no genetic mechanisms explaining this

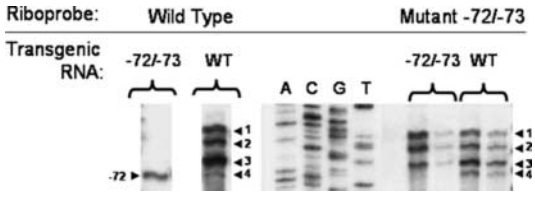

Figure 4. Transcription initiation site mapping in reticulocyte RNA from transgenic mice. Wild-type riboprobe (left). Ribonuclease protection with the wild-type riboprobe and RNA from −72/−73 mutant promoter transgenic mice identified a single, truncated protected fragment, demonstrating the presence of the deletion in these animals. Hybridization to RNA from wild-type promoter transgenic mice identified four transcription initiation sites labeled 1–4. Each lane represents RNA from a separate transgenic line. Mutant −72/−73 riboprobe (right). Ribonuclease protection with the mutant −72/−73 riboprobe and RNA from −72/−73 mutant promoter transgenic mice detected only three transcription initiation sites compared with four in the wild-type promoter transgenic mice, with significant differences in relative transcript expression.

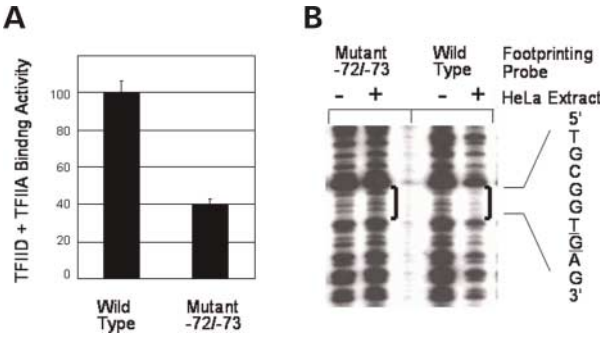

Figure 5. *In vitro* analyses of DNA–protein interactions. (**A**) Probe binding and complex formation in magnesium agarose gel electrophoresis. Wild-type and mutant −72/−72 ^{32}P-labeled ankyrin gene promoter fragments from −296 to −15 were used as probes in magnesium agarose gel electrophoresis assay. When compared with wild-type, the mutant probe exhibited reduced binding with a combination of recombinant TFIIA and recombinant TFIID under the same conditions, 39.9 ± 4.7% ($P = 0.0002$) of control. (**B**) *In vitro* DNase I footprinting. *In vitro* DNase I footprinting was performed with wild-type and mutant ankyrin core promoter probes and recombinant TFIID (TBP) protein. A region from −68 to −75 was protected with a wild-type promoter probe (left), which was not protected when a mutant −72/−73 promoter probe (right) was used.

variability in ankyrin-linked HS have been described (30). Variable penetrance, *cis* or *trans* ankyrin gene variants and modifier genes have been hypothesized. In this German kindred, the proband is a compound heterozygote for the −72/−73 promoter mutation on one allele and the exon 6 frameshift mutation on the other allele. Both parents have mild-to-moderate HS, i.e. with compensated hemolysis and reticulocytosis, and like many HS patients, came to attention only during family studies.

In most protein-encoding genes, initiation of RNA polymerase II-dependent transcription requires assembly of numerous proteins at the core promoter forming a preinitiation complex. Recruitment of TFIID, a multiprotein complex, composed of the TBP and associated proteins TAFs (TBP-associated factors), is the first step in preinitiation complex formation (31–33). TFIID recognizes and binds the core promoter through interactions of TBP with the TATA box, the InR and/or the DPE (32,34–39). In core promoters lacking conserved *cis* elements, Sp1 directs formation of preinitiation complexes to a region 40–100 bp downstream of its binding sites (7,20,34).

We have previously shown the functional importance of two Sp1 sites positioned 80–100 bp upstream of the core ankyrin promoter in directing gene expression (Fig. 6) (18). Mutation or deletion of these Sp1 sites decreases or abolishes promoter function. It has been hypothesized that in core promoters lacking conserved *cis* elements, preinitiation complexes are formed via binding of the TBP subunit of the TFIID complex to promoter DNA sequences that most closely resemble functional TATA-like or InR elements (20). If multiple sequences are similarly desirable for recognition, multiple start sites will result, as observed with the ankyrin promoter. *In vitro* studies suggest that alterations within the start site region may lead to relocalization of the start sites and variation in promoter strength (40). It has been speculated that the variation in promoter strength, as observed in this case, is due to alteration in promoter context, an area of growing importance in promoter function (15,34). There has been growing awareness of the importance of promoter We observed alterations in start site utilization and reporter gene expression *in vitro* and *in vivo*, suggesting disruption of preinitiation complex formation by the −72/−73 mutation. As hypothesized (20), the wild-type transcription initiation site interacted with the TFIID complex *in vitro* and deletion of this site disrupted this interaction. These studies indicate that the human wild-type ankyrin gene erythroid promoter not only shares the structure of the class of *Drosophila* core promoters lacking conserved *cis* elements but also shares the functional characteristics including critical upstream Sp1 sites. Our study is the first to demonstrate altered gene expression and start site utilization causing a disease phenotype by mutation in a core promoter lacking conserved *cis* elements.

The lack of functional studies associated with sequence variation in core promoters lacking conserved *cis* elements precludes comment regarding the frequency of these mutations. Similar to what we observed, a mutation in the ubiquitous promoter of the porphobilogen deaminase (HMBS) gene (deletion of a nucleotide immediately 5′ to one of the transcription initiation sites), associated with the non-erythroid form of acute intermittent porphyria, yielded results strikingly similar to ours (41). The HMBS gene promoter is C+G rich, lacks conserved *cis* elements and has multiple transcription initiation sites. When compared with our acrylamide gel EMSA, a wild-type HMBS probe yielded a very high molecular weight complex that was absent when a mutant probe was used. Functional studies, including protein binding studies and *in vivo* analyses of promoter function, were not performed. We hypothesize that the HMBS mutation would also lead to decreased gene expression and altered start site utilization. We predict that variations in core promoter sequences are an under-recognized cause of other inherited disorders also.

MATERIALS AND METHODS

Patients

The proband was from a German HS kindred. Diagnostic criteria for HS were based on typical clinical features, the

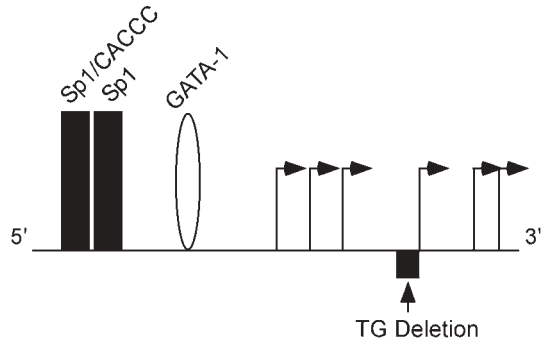

Figure 6. The ankyrin gene erythroid promoter. The critical Sp1, Sp1/CACCC and GATA-1 sites are shown. The previously identified transcription initiation sites are denoted by the arrows. The location of the TG deletion is denoted by the box.

presence of spherocytes on peripheral blood smear, increased incubated osmotic fragility and a negative direct antiglobulin test. The proband has moderate HS and was splenectomized in childhood. Postsplenectomy, the hemoglobin is 12.3 g/dl and the reticulocyte count is 14.2%. Both parents have normal hematocrits, increased reticulocyte counts and abnormal erythrocyte incubated osmotic fragility, typical for the diagnosis of HS with compensated hemolysis (Table 1).

Erythrocyte membrane preparation and quantitation

Erythrocyte membranes were prepared from whole blood as described previously (42). Membrane proteins were separated by SDS–PAGE in 4–16% gradient polyacrylamide gels and stained with Coomassie blue. Ankyrin/band 3 and spectrin/band 3 ratios were obtained by densitometric scanning of the stained gels as described (42).

Mutation detection

Genomic DNA from the proband and mother was amplified by PCR using two pairs of primers flanking the ankyrin gene erythroid promoter region and exon 6, respectively, as described (Table 4) (1). The father is deceased and no genetic material was available for study. SSCP analysis was performed according to Eber *et al.* (1). SSCP was repeated on samples with abnormal patterns on initial screening. Genomic DNA fragments demonstrating a consistently abnormal SSCP pattern were amplified in a second PCR reaction, subcloned and subjected to nucleotide sequence analysis.

Transfection analyses

We have previously shown that a 286 bp minimal ankyrin gene promoter fragment directs high-level expression of a luciferase reporter gene in erythroid cells (18). A −72/−73 mutant ankyrin promoter fragment corresponding to this 286 bp promoter fragment generated by PCR amplification was subcloned into the firefly luciferase reporter plasmid pGL2B (Promega Corp., Madison, WI, USA) to yield p296 − 72/−73. Test plasmids, p296 and p296 − 72/−73, were sequenced to exclude cloning or PCR-generated artifacts. Transient transfection analyses were performed as described (18).

In vitro transcription

Cell-free *in vitro* transcription was performed using a run-off technique (HeLaScribe, Promega, Madison, WI, USA) with K562 or HeLa cell nuclear extracts as described (43). Templates for the reaction were the wild-type or the mutant −72/−73 ankyrin core promoter fragments in pGL2B containing both upstream Sp1 binding sites linearized with *Sfu*I or a positive control template that contains the CMV immediate early promoter. For each reaction, 200 ng of template was used. Final $MgCl_2$ concentration in each reaction was 3 mM. After incubation at 30°C for 60 min, reactions were stopped and ethanol precipitated. After adding loading dye, run-off transcription products were analyzed by slot blot analysis or on a denaturing polyacrylamide gel.

Preparation of mutant promoter–reporter constructs for transgenic mice

To generate a mutant −72/−73 ankyrin promoter/$^{A}\gamma$-globin transgene, a 276 bp *Sma*I/*Bgl*II fragment containing the mutant ankyrin promoter (−291 to −20 plus polylinker sequence) was excised from the pGL2B luciferase reporter vector. A 1938 bp *Bgl*II/*Hin*dIII $^{A}\gamma$-globin fragment was excised from plasmid 72βsp$^{A}\gamma$ (29). A triple ligation consisting of the ankyrin promoter, the $^{A}\gamma$-globin gene and *Sma*I/*Hin*dIII digested pSP72 was used to generate the mutant −72/−73 ankyrin/$^{A}\gamma$-globin plasmid. The ankyrin/$^{A}\gamma$-globin gene fragment (2244 bp) was excised from the plasmid with *Eco*RV and *Hin*dIII and transgenic mice generated and analyzed as described (44).

Isolation of RNA and RNase protection assays (RPAs)

Total cellular RNA was extracted from adult reticulocytes, obtained by collecting 200 μl of blood from phlebotomized animals, as described (44). Three different riboprobes were used for RPA. The first riboprobe contains sequences for both exon 2 of the human $^{A}\gamma$-globin gene and exon 2 of the murine α-globin gene (28,45), ensuring that the human $^{A}\gamma$-globin and murine α-globin sequences are labeled to equal specific activity, allowing direct comparison of human $^{A}\gamma$-globin and murine α-globin mRNA levels. To map the transgene mRNA start sites, riboprobes were generated by digesting either the wild-type or the −72/−73 mutant ankyrin promoter/$^{A}\gamma$-globin transgenes described previously with *Bst*EII (located in exon 1 of the γ-globin gene) and *Hin*dIII (3′ end of the transgene), blunting the ends and religating. The linear templates protect exon 1 of the γ-globin gene and extend through the ankyrin promoter region. Fragments protected from RNase digestion were run in lanes adjacent to a sequencing ladder generated from a wild-type ankyrin promoter/$^{A}\gamma$-globin transgene template with a primer corresponding to the 3′ end of γ-globin exon 1 (5′-CCTTCCCAGGGTTTCTC-3′). Probe preparation, hybridization, RNA digestion and electrophoresis were carried out as described (28,29). To quantitate the levels of mRNA or the relative amounts of each transcript, the gel was exposed to a phosphorimager screen and scanned on a molecular dynamics phosphorimager.

Electrophoretic mobility shift analyses (EMSA)

Nuclear extracts were prepared as described (46). Standard acrylamide gel EMSAs were carried out as described using oligonucleotide primers as probes (Table 2) (18). Magnesium agarose EMSAs were performed exactly as described (47). Wild-type or mutant −72/−73 ankyrin probes excised from pGL2B plasmids with *Sma*I and *Hin*dIII were end-labeled with [γ-^{32}P]ATP. Labeled promoter fragments were incubated with recombinant human TBP (Santa Cruz) alone or with recombinant TFIIA (Austral Biologicals, San Ramon, CA, USA) and/or recombinant TFIIB (Santa Cruz). In the binding reactions, the amounts of recombinant proteins were titrated over a range of concentrations (TBP and TFIIB both 66–660 ng and TFIIA 2–20 ng per reaction). The final KCl concentration in each reaction mix was 60 mM. Reaction products were electrophoresed at room temperature in 1.4% magnesium agarose gels. The gels were dried and subjected to autoradiography, followed by exposure to a phophorimager screen and quantitative analyses on a molecular dynamics phosphorimager.

In vitro DNase I footprinting

In vitro DNase I footprinting was performed as described (18) with the same wild-type and mutant ankyrin promoter probes used in the magnesium agarose EMSA as templates. Footprinting reaction mixes contained HeLa cell nuclear extracts, recombinant TBP alone or a mixture of recombinant TBP and recombinant TFIIA.

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