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Analysis of severe, atypical hereditary spherocytosis (HS) associated with combined spectrin and ankyrin deficiency and mental retardation.

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ANALYSIS OF SEVERE, ATYPICAL HEREDITARY SPHEROCYTOSIS (HS) ASSOCIATED WITH COMBINED SPECTRIN AND ANKYRIN DEFICIENCY AND MENTAL RETARDATION

by

FOTIOS TSOUMANIS

A DISSERTATION

Submitted in partial fulfillment of the requirements for
the degree of Doctor of Philosophy in the Graduate Program in
Medical Genetics in the Graduate School,
The University of Alabama at Birmingham

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ABSTRACT OF DISSERTATION
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Title Analysis of Severe, Atypical Hereditary Spherocytosis (HS) Associated with Combined Spectrin and Ankyrin Deficiency and Mental Retardation.

Hereditary Spherocytosis (HS) is one of the most common forms of congenital hemolytic anemia. The primary molecular defects lie in the red blood cell (RBC) membrane skeleton components, and identified lesions include mutations of spectrin, ankyrin, band 3 protein, and protein 4.2. Coetzer and co-workers previously reported a patient with combined deficiency of spectrin and ankyrin with severe anisocytic HS and CNS abnormalities (Coetzer et al. 1988). The primary defect underlying HS in this patient was determined to be a decrease of the erythrocyte ankyrin mRNA transcript while both spectrin mRNA transcripts were normal (Hanspal et al. 1991). We have engaged in studies to clarify the nature of the molecular defect(s) underscoring HS in this patient. Using the 5'- and 3'-RACE techniques, reticulocyte RNA was analyzed to investigate the possibility of a mutational event in those regions of the erythrocyte ankyrin gene. Evidence of alternative mRNA processing was searched for to explain the substantially increased ratio of the two ankyrin transcripts in the propositus. No specific differences between the patient and normal controls were revealed. Subsequently, scanning of the entire erythrocyte ankyrin cDNA for mutations by SSCP analysis was completed. PCR-amplified cDNA from the propositus had been used and compared to normal controls. Two abnormally migrating fragments were identified. Further analysis and characterization of these two fragments, including subcloning and sequencing, did not reveal any DNA changes in their primary nucleotide sequence. The possibility of null or trace allele mutations was subsequently investigated, by taking advantage of a previously reported exonic $(AC)_n$ repeat

polymorphism in the 3'-UTR of ankyrin mRNA and the report that comparing the ankyrin $(AC)_n$ microsatellites in genomic DNA and cDNA could be used to study erythroid ankyrin expression (Polymeropoulos et al. 1991; Jarolim et al. 1995b). Heterozygosity for the ankyrin AC repeat length both in genomic DNA and cDNA was demonstrated. This was indicative of the fact that both ankyrin alleles were transcribed normally, thereby ruling out the possibility of a null mutation or trace amount allele, as was originally hypothesized (Hanspal et al. 1991). The causative mechanism(s) responsible for reduced stability or increased susceptibility to proteolytic degradation of the ankyrin mRNA transcript are as of yet unidentified. Erythrocyte ankyrin (ANK1) has been known to also be expressed in brain; in light of this finding, the clarification of the molecular defect of this unusually severe, transfusion-dependent, anisocytic spherocytosis in this patient with CNS abnormalities, could shed light on those functionally important domains of ankyrin that are shared by neurological tissues and erythrocytes.

Abstract Approved by: Committee Chairman _____

Program Director _____

Date 6/18/96 Dean of Graduate School _____

DEDICATION

With endless love, to my beloved and respected parents, Georgios and Chrysoula Tsoumanis, for their unconditional love, continued support, and encouragement, without which my studies would not have been accomplished.

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LIST OF ABBREVIATIONS

AMV	avian myeloblastosis virus
ANK	ankyrin peptide(protein designation)
Ank	human ankyrin (gene designation)
Ank	mouse ankyrin (gene designation)
ASPCR	allele-specific polymerase chain reaction
bp	base pair
BFU-E	blast forming unit-erythroid
cDNA	coding deoxyribonucleic acid
CFU-E	colony forming unit-erythroid
CNS	central nervous system
dATP	deoxy-adenosine triphosphate
dCTP	deoxy-cytosine triphosphate
dGTP	deoxy-guanine triphosphate
dH_2O	distilled water
dITP	deoxy-inosine triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxy-nucleotide triphosphate
DTT	dithiothreitol
FISH	fluorescent in situ hybridization
FVA	friend virus anemia
fl	femtoliter
HE	hereditary elliptocytosis
HEL	human erythroleukemia

HPP	hereditary pyropoikilocytosis
HS	hereditary spherocytosis
kb	kilobase
kDa / kd	kiloDalton
M-MLV H$^-$	murine-moloney leukemia virus
MCV	mean corpuscular volume
MI / CMM	million / ml
mRNA	messenger ribonucleic acid
MW	molecular weight
nb	normoblastosis gene
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
RACE	rapid amplification of cDNA ends
RBC	red blood cell
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
SAO	southeastern asian ovalocytosis
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SPH-1	spherocytosis locus-1
SPH-2	spherocytosis locus-2
SSCP	single strand conformational polymorphism
TBE	Tris-boric acid-EDTA
TE	Tris-EDTA
UTR	untranslated region
VA	Veterans Administration

INTRODUCTION

Overview of the Erythrocyte Membrane

The erythrocyte membrane is composed of a lipid bilayer to which is anchored a filamentous network of proteins that underlies the cytoplasmic surface of the membrane (fig. 1). This network is responsible for maintaining the biconcave disc shape, mechanical stability, and cellular deformability of the erythrocyte (fig. 2). The major components of this complex cytoskeletal latticework of proteins include spectrin, ankyrin (protein 2.1), protein 4.1, and actin (Mohandas and Chasis 1993). The numerals (2.1, 4.1, etc.) designate the electrophoretic mobility of each protein on sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE), first described by Fairbanks (Fairbanks et al. 1971) (table 1).

Spectrin is the major component, accounting for about 70% of the cytoskeletal protein mass. Erythroid spectrin is a fiber-like protein present in red cells at approximately 200,000 copies per cell. It is composed of two subunits, α (240 kd) and β (220 kd), that are structurally distinct and are encoded by separate genes. A and β spectrin chains intertwine in an antiparallel orientation with respect to their amino-termini, to form flexible heterodimers, which in turn self-associate at their head region to form $(\alpha\beta)_2$ tetramers and, to a lesser extent, higher-degree oligomers (Shotton at al. 1979). These oligomers are interconnected into a two-dimensional network by their linkage to actin subunits. This interaction is greatly promoted by protein 4.1 through direct contact with spectrin at sites close to the region in which spectrin reacts with actin. Protein 4.1 also binds to glycophorins A and C, two integral proteins that span the lipid bilayer. Glycophorin C seems to be an important attachment site bearing a critical role in regulating the stability,

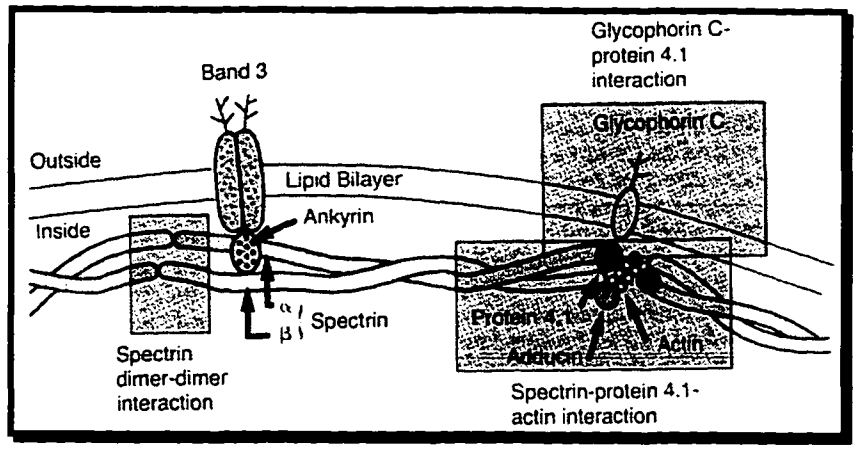

Figure 1 Schematic diagram of the red cell membrane organization based on the current understanding of various protein interactions. The key protein interactions identified to be important in regulating red cell membrane mechanical stability include spectrin dimer-dimer interaction, spectrin-actin-protein 4.1 interaction, band 3-ankyrin-spectrin interaction, and glycophorin C-protein 4.1 interaction. Adducin, a membrane skeleton-associated calmodulin-binding protein, also promotes spectrin-actin interaction. (From Mohandas 1995)

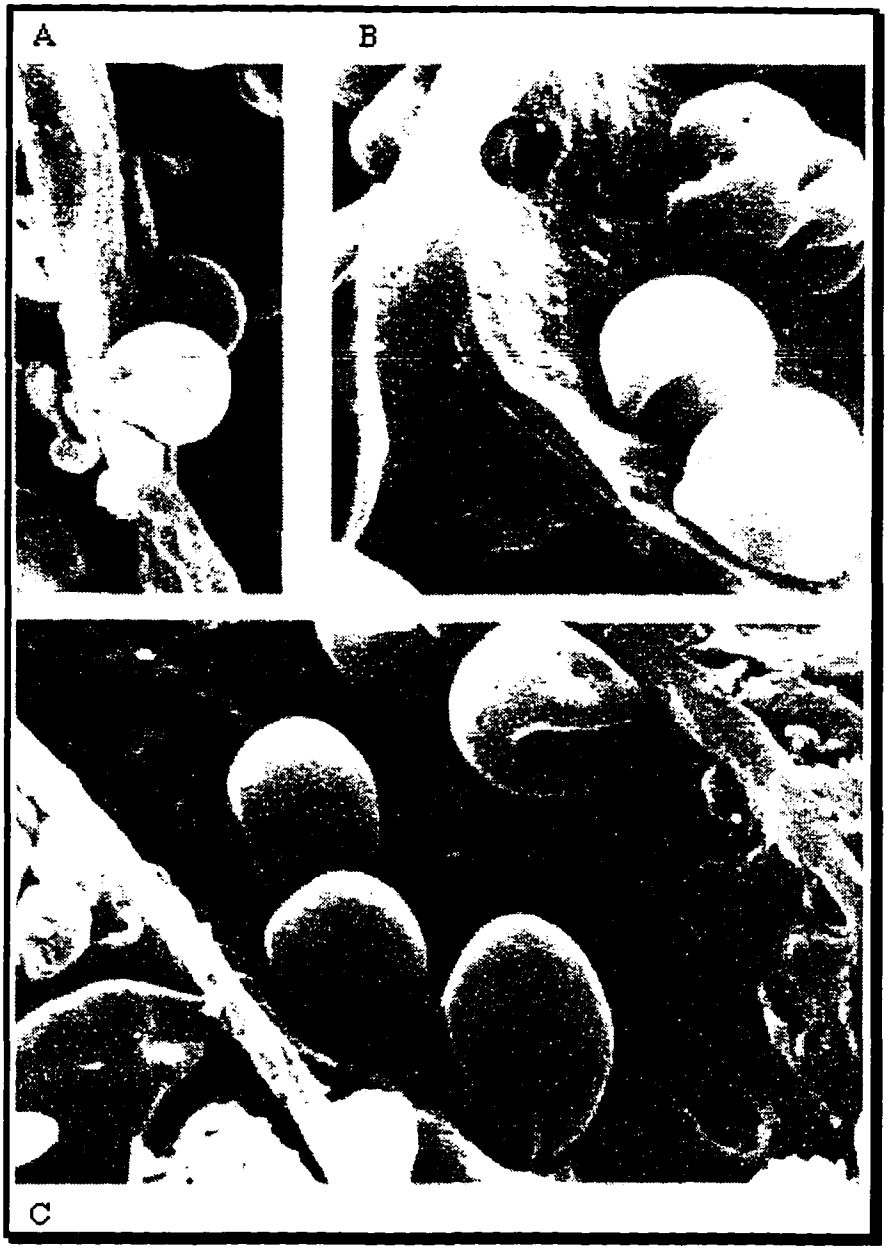

Figure 2 Scanning electron micrographs of red blood cells passing through splenic endothelial slits. Note the remarkable deformation exhibited by the red cells during their transit through these structures. The cell with the lobular and irregular surface in (B) is a spherocyte in transit. (From Mohandas 1995)

Table 1

Major Red Blood Cell Membrane Proteins and Their Involvement in Hereditary Hemolytic Anemias. (Palek and Jarolim 1995)

band	protein	MW(gel) (kd)	MW (calc) kd	chromosome localization	gene size (kb)	No. of exons	involvement in anemia
1	α-spectrin	240	280	1q22-q23	80	52	HE, HS
2	β-spectrin	220	246	14q23-q24.2	>100	~32	HE, HS
2.1	ankyrin, 2.1 isoform [a]	210	206	8p11.2	>100	40	HS
3	band 3 protein	90-100	102	17q21-qter	17	20	HS, SAO, HAc
4.1	protein 4.1	80	66	1p33-p34.2	>100		HE
4.2	pallidin	72	77	15q15-q21	20	13	HS
5	β-actin	43		7pter-q22			N
PAS-1	glycophorin A	36		4q28-q31	>40	7	N
PAS-2	glycophorin C	32	14	2q14-q21	14	4	HE

Abbreviations: HS, hereditary spherocytosis; HE, hereditary elliptocytosis; HPP, hereditary pyropoikilocytosis; SAO, Southeast Asian ovalocytosis, HAc, hereditary acanthocytosis; HSt, hereditary stomatocytosis; N, no hematologic abnormalities reported; [a] Bands 2.1, 22, 2.3, , and 2.6 are protein isoforms of erythroid ankyrin, at least some of which are produced by alternative ankyrin mRNA processing.

deformability, and shape of the membrane. Its deficiency is associated with protein 4.1 deficit and reduced membrane mechanical stability.

The task of physically linking the spectrin lattice to the plasma membrane falls mainly onto two proteins, ankyrin (band 2.1) and protein 4.1. Ankyrin is the primary membrane skeleton linker molecule, attaching the spectrin tetramers to the cytoplasmic domain of protein 3, also known as the transmembrane anion exchange channel. Band 3 is a glycoprotein of 100 kDa in molecular mass. The importance of ankyrin to the integrity of the erythrocyte is exemplified by an ankyrin-deficient form of murine hereditary spherocytosis (HS), the normoblastosis mutation (gene symbol *nb*). Moreover, lesions in human erythrocyte ankyrin can cause HS and severe hemolytic anemia (Peters and Lux 1993).

Clinical Expression of Erythrocyte Membrane Protein Mutations

Genetic defects of the membrane skeleton components are known to compromise the integrity of the erythrocyte membrane, producing mechanically fragile, prone to fragmentation, abnormally shaped erythrocytes that are removed by the spleen ("splenic conditioning"). Inherited red cell abnormalities that are attributable to primary genetic defects in membrane proteins were originally classified based on their morphological presentation, leading to at least five distinct phenotypes: (i) hereditary spherocytosis (HS), (ii) hereditary elliptocytosis and pyropoikilocytosis (HE and HPP), (iii) Southeast Asian Ovalocytosis (SAO), (iv) hereditary acanthocytosis (HAc), and (v) hereditary stomatocytosis (HSt). Approaches to the detection of the underlying molecular defect usually involve membrane protein analysis by gel electrophoresis followed by direct quantitation of membrane proteins. The next step in the elucidation of these defects is frequently more complex and may include studies of protein function, biosynthetic studies, and screening for the presence of point mutations, small deletions, or both. RFLP linkage studies (either for linkage exclusion or, in large families, for linkage verification) have led to the clarification of the underlying defects in certain kindreds (Palek and Jarolim 1993).

HS, the most common among these conditions, is both clinically and genetically heterogeneous with both autosomal dominant or recessive modes of inheritance. Figure 3 shows scanning electron micrographs of spherocytes from patients with HS. Bodine and co-workers first characterized several mouse strains with HS, which exhibited a spectrin deficiency relative to other proteins of the membrane skeleton (Bodine et al. 1984). Closer analysis revealed that in some mouse strains the spectrin deficiency was due to abnormalities in either α- or β-spectrin, while in another strain a decrease in ankyrin synthesis underlay HS. It was later reported that a deficiency of spectrin is present in most patients with HS (Agre et al. 1985). The initial study of the proband in our pedigree in 1987 contributed to our current understanding of spherocytosis, in that a deficiency of spectrin at the protein level may actually be a secondary defect. Since then, a number of

additional membrane protein abnormalities have been reported (table 2), (Palek and Jarolim 1993). HE and HPP, as separate entities, are likewise heterogeneous with regards to clinical severity, inheritance, and the underlying molecular defects. Common HE and its most severe form, HPP, are characterized by unstable RBCs that are prone to fragmentation in vivo. The underlying genetic defects involve mutations of spectrin, deficiencies of protein 4.1 or, less frequently, glycophorin C. It is now known that HPP represents a subset of HE rather than a distinct disorder. Identical α-spectrin mutations have been identified in both disorders, but the defect is more severe in HPP where it is present in conjunction with a partial spectrin deficiency. HPP is virtually indistinguishable from homozygous HE. The only feature that distinguishes them is the presence of microspherocytosis and a partial deficiency of spectrin. Coexistence of both disorders (HE-HPP) has been reported in the same kindred (Palek 1987). SAO is highly prevalent in southeast Asian subpopulations and involves molecular defects of the band 3 protein. Membrane protein mutations that are associated with acanthocytosis or stomatocytosis are very poorly understood.

Human Hereditary Spherocytosis

HS is a familial hemolytic disorder characterized by anemia, intermittent jaundice, splenomegaly, and a favorable responsiveness to splenectomy. Morphologically, its hallmark is the microspherocyte in the peripheral smear (fig. 3). Although it is primarily transmitted in an autosomal dominant mode, it may remain clinically silent for indefinite periods. Consequently, the true extent of familial involvement can only be evaluated after thorough hematologic examination of family members (Wintrobe et al. 1981).

The earliest clinical account of HS was that of Vanlair and Masius, who in 1871 described a disorder they called "microcythémie." HS was rediscovered 20 years later by Wilson and Minkowski (Palek and Jarolim 1995). The next major contributions by Haden and by Castle and Daland drew attention to a potential structural involvement as the underlying basis for hemolysis. The ultimate molecular lesion in HS is yet unidentified, but

Table 2

Molecular Defects of the Red Blood Cell Membrane Proteins Associated with HS. (Palek and Sahr 1992)

protein	biochemical phenotype	structure / function defect	molecular defect	inheritance
α-spectrin	severe spectrin deficiency	abnormal tryptic digest of the αII domain	linked to α-spectrin polymorphism	recessive
β-spectrin	partial spectrin deficiency	weak interaction of spectrin with protein 4.1	201Trp→Arg	dominant
ankyrin	deficiency of spectrin and ankyrin	unidentified	approximately 50% reduction in ankyrin mRNA	not established
ankyrin Prague	partial ankyrin deficiency	deficiency of ankyrin 2.1. Additional 174 kDa ankyrin (2.2')	abnormality in the regulatory domain of ankyrin	dominant
band 3 Prague	partial band 3 deficiency (30%)	unidentified	duplication of 10 bases following codon 818	dominant
band 3 Tuscaloosa	partial protein 4.2 deficiency (30%)	30% decrease in binding of protein 4.2 to inside-out-vesicles	327Pro→Arg and a linked polymorph-ism 56Lys→Glu	dominant
protein 4.2	doublet of 72 and 74 kDa by immunoblotting	unidentified	142Ala→Thr	not established

its definition would afford important insight into the molecular biology and function of red cell membranes (Palek and Lambert 1990).

HS has been recognized as one of the most common hemolytic anemias with a tendency towards a higher frequency in people of northern European descent, although it has been known to occur sporadically in all ethnic groups, such as African and Japanese populations (Palek and Jarolim 1995). This apparent preponderance may actually reflect an observer bias, because the disease can go unnoticed without extensive testing. The patterns of inheritance of HS are quite complex. The majority of the cases have been inherited in an autosomal dominant mode; the remainder were either autosomal recessive traits or paradigms of new mutations or had reduced penetrance, variable expressivity, or both (Palek and Jarolim 1995). Only a few cases of homozygous HS have been reported and were associated with severe hemolytic anemia. It appears that homozygosity of the more typical hemolytic form of HS is most likely lethal. The clinical spectrum of HS has been

quite variable including mild and asymptomatic forms, as well as severe forms. The prevalence of HS in the United States is 1 in 5,000 (Palek and Jarolim 1995).

The primary molecular defects lie in the membrane skeleton, in proteins that attach the skeleton to the overlying bilayer of lipids and integral membrane constituents. These are the proteins that form the major "vertical" interactions of the skeleton. These protein-protein and protein-lipid interactions have been reported to stabilize the lipid bilayer membrane. They have involved the spectrin-ankyrin-band 3 interaction, the protein 4.1-glycophorin C linkage and the weak interactions between the skeletal proteins and the negatively charged lipids of the inner half of the membrane lipid bilayer (Palek and Jarolim 1995).

Defects in these proteins fit with the predominant theory that HS is caused by the local disconnection of the skeleton and the lipid bilayer, followed by vesiculation of the unsupported surface components. This, in turn, leads to a progressive reduction in the membrane surface area ("spherocytosis") impairing cellular deformability, which leads to the entrapment of the nondeformable spherocytes in the fenestrations in the wall of splenic sinuses. Once trapped in the spleen, HS RBCs undergo further damage as manifested by additional loss of surface area and are eventually destroyed. The spleen appears to be an active protagonist in the pathophysiology of the disease and not just a passive filter. It aggravates the basic membrane molecular defect and expedites membrane loss, a process known as "splenic conditioning" (fig. 4). Any molecular elucidation of HS must eventually lead to an understanding of this complex phenomenon (Peters and Lux 1993).

The membrane skeletal defects in HS have been characterized by genetic and protein-based approaches. Two spherocytosis loci, SPH1, located on chromosome 14q24.1, and SPH2, on chromosome 8p11.2, have been identified by cytogenetic and linkage studies. Additionally, it has been shown that HS can be caused by spectrin, protein 3 or protein 4.2 deficiency, or, quite commonly, by a combined deficiency of both spectrin and ankyrin (Peters and Lux 1993).

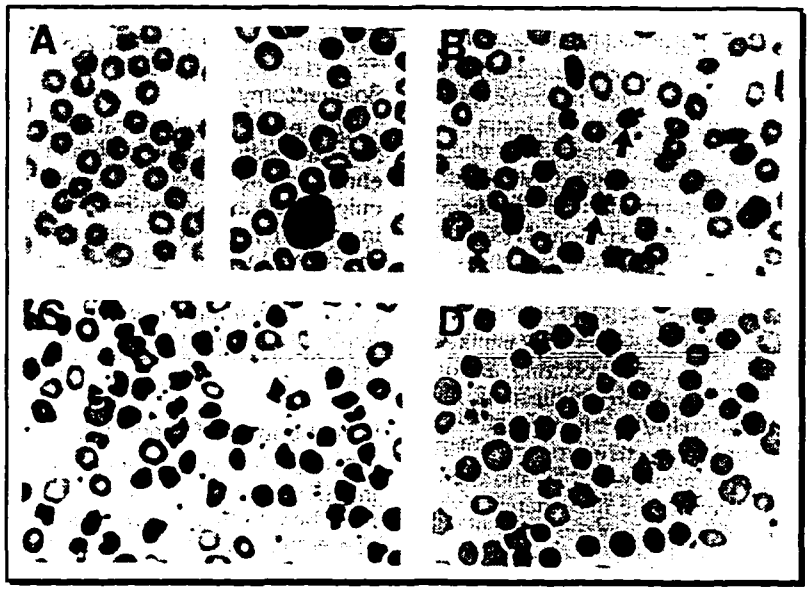

Figure 3 Blood films from patients with HS of varying degrees. (A) Two blood films of typical moderately severe HS with a mild deficiency of red cell spectrin and ankyrin. Although many cells have spheroidal shape, some retain a central concavity. (B) HS with pincered red cells (arrows), as typically seen in HS associated with band 3 deficiency. Occasional spiculated red cells are also present. (C) Severe atypical HS due to a severe combined deficiency of spectrin and ankyrin. This is the actual blood film of the propositus in the pedigree under study. In addition to spherocytes, there are many anisocytic cells, with irregular contour. (D) HS with isolated spectrin deficiency due to ab-spectrin mutation. Some of the spherocytes have prominent surface projections resembling spheroacanthocytes. (From Palek and Jarolim 1995)

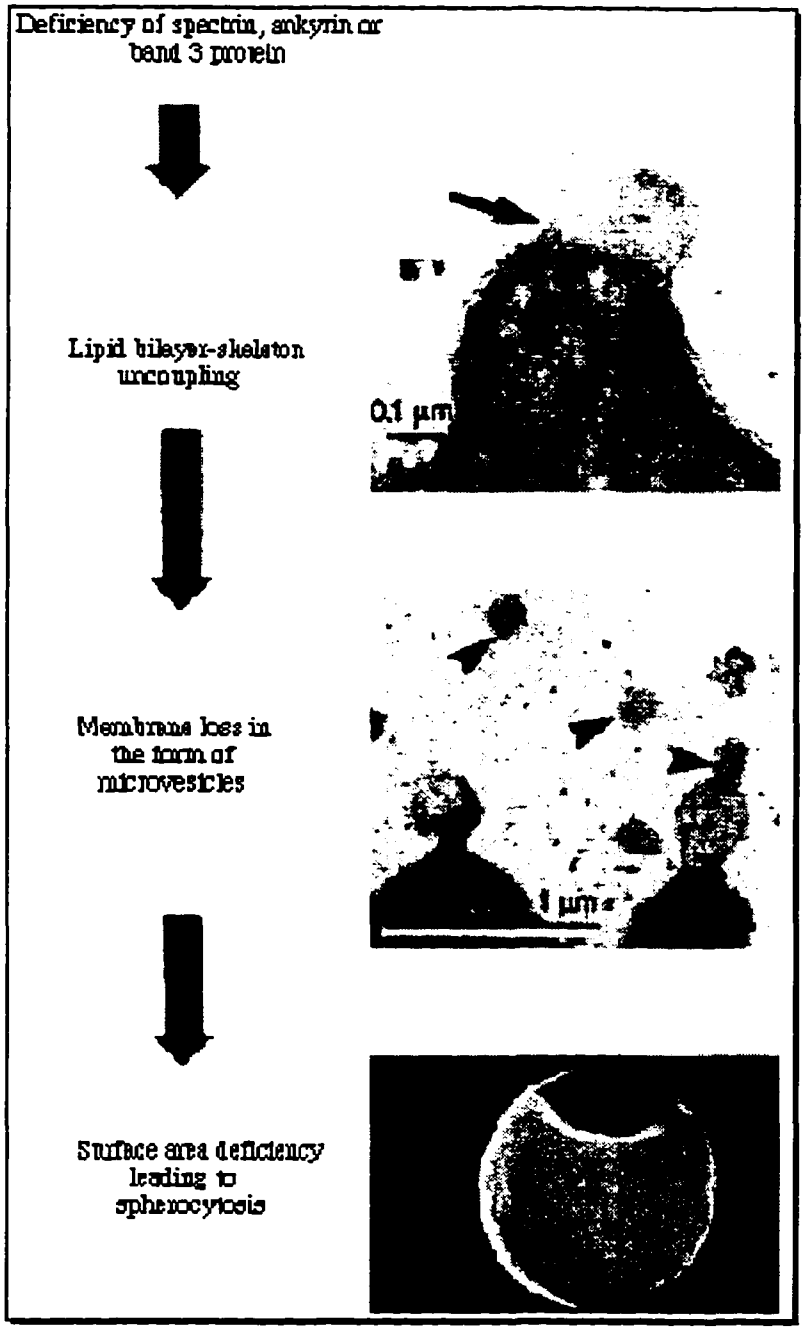

Figure 4 Vertical interactions of membrane proteins and the pathobiology of the red cell lesion in hereditary spherocytosis. Partial deficiencies of spectrin, ankyrin, or band 3 protein lead to uncoupling of the membrane lipid bilayer from the underlying skeleton (arrow) followed by formation of spectrin-free microvesicles of about 0.2-0.5 µm in diameter (arrowheads). These can be visualized by transmission electron microscopy, but they are not seen during examination of a peripheral blood film. The subsequent loss of cell surface area and a decrease in the surface/volume ratio leads to spherocytosis. (From Palek and Jarolim 1995)

In the pedigree under study (shown in Results, Family Pedigree), the propositus was reported to have a combined deficiency of spectrin and ankyrin associated with severe anisocytic HS and CNS involvement (Coetzer et al. 1988). Erythrocyte spectrin and ankyrin levels were half normal, and further analysis determined the primary molecular defect underlying HS to be an ankyrin deficiency, either failure of ankyrin synthesis or synthesis of an unstable ankyrin molecule (Hanspal et al. 1991); this was the seminal first paradigm of a primary deficiency of ankyrin underlying a secondary spectrin deficiency manifested in HS.

Ankyrin(s)

Ankyrin (after the Greek ankyra, anchor) was discovered during a search for the membrane spectrin attachment site on the erythrocyte membrane. The ankyrins are a family of related genes that probably arose by duplication and divergence of a common ancestral gene. Two ankyrin genes have been cloned, sequenced, and mapped in both the human and mouse systems: erythrocyte ankyrin is on chromosome 8 (recently mapped to 8p11.2 by Fluorescence In Situ Hybridization [FISH]) in both humans (gene symbol ANK1) and mouse (gene symbol *Ank*-1). The major form of ankyrin in the brain is biochemically similar to erythrocyte ankyrin and is encoded by a gene designated ANK2 on human chromosome 4q25-q27 and *Ank*-2 on mouse chromosome 3.

Additionally, a third ankyrin gene has been identified in several mouse tissues, including brain, kidney, muscle, skin, liver, and thymus, with similarity to ANK1 and ANK2, but it is clearly distinct from each. It is likely that other ankyrin genes exist, although probably not a large number (table 3, Peters and Lux 1993). Based on the nature of the various ankyrin ligands (table 3), it has appeared that ankyrins may function as adaptors that mediate and possibly regulate the various interactions between constituents of the cytoskeleton and a variety of integral membrane components such as transporters, receptors, or adhesive proteins (Peters and Lux 1993).

Structural and Functional Domains of Erythrocyte Ankyrin (Ank1)

Human erythrocyte ankyrin is actually a mixture of proteins of different size. The major form (protein 2.1), a 206-kd (1,880 amino acids) protein, migrated at 210 kd on SDS gels. Other faster migrating ankyrin species, designated as proteins 2.2 (186-kd), 2.3 (170-kd), and 2.6, were easily detected by standard protein stains. Up to eight less-abundant ankyrins were visualized on immunoblots with high titer antisera, extending from 320 to 117 kd. Recent molecular analyses indicated that protein 2.2 was a product of alternative splicing, which may also be the case for the rest of the ankyrin isoforms (Peters and Lux 1993).

Table 3

Ankyrins. (Palek and Sahr 1992)

GENES	EXPRESSED	INTEGRAL PROTEINS BOUND	SKELETAL PROTEINS BOUND
Erythroid ankyrin (Ank1)	Red blood cells Purkinje cells Muscle cells Endothelial cells	Red cell anion exchanger (AE1) GP116 (CD44-like endothelial protein)	Spectrin Protein 4.2 Tubulin Vimentin
Brain ankyrin (Ank2)	Most neuron Glial cells	Ankyrin binding glycoprotein 205	Fodrin Tubulin
??Additional brain ankyrin gene(s)	Nodes of Ranvier Axon hillcock Unmyelinated axons	Na channel (voltage dependent)	N/A
Epithelial ankyrin(s)	Basolateral surfaces of kidney	Na, K-ATPase AE1 (kidney)	Fodrin
Lymphocyte ankyrin	T and B cells	CD44	Fodrin

Erythrocyte ankyrin is composed of three functional domains as defined by chymotrypsin cleavage (fig. 5A and B). The N-terminal 89-kd protein 3-binding domain extends from Pro 2 to Phe 827. The central, 62-kd spectrin-binding domain starts at Lys 828 and extends to either Leu 1,382, or Tyr 1,386. The remaining 495-to-499 C-terminal amino acids constitute a functional domain (55-kd) that has been found to regulate the binding of ankyrin to spectrin and the anion-exchange protein (Peters and Lux 1993).

There is evidence supporting the idea that this regulatory domain varied in sequence from ankyrin to ankyrin and was subject to alternative splicing. During sequencing, two areas of alternative splicing were discovered. One was responsible for ankyrin isoform 2.2 (band 2.2), which resulted from the loss of the domain between Gly 1,513 and Gly 1,675. Protein 2.2 was created by the use of an alternate acceptor splice site within a large exon leading to the in-frame deletion of 162 amino acids. The second region of alternative splicing involved splicing of three exons at the C-terminus. The spliced portions appear to have regulatory functions. Ankyrin 2.2 has a 3-fold higher affinity for spectrin, the ability to bind twice as many band 3 molecules, and 5- to 10- fold more sites on kidney membranes. In general, the 2.1 insert acts as a repressor, although the mechanism of this effect is not understood. Undoubtedly, this domain bears significance in the dynamics of ankyrin function, and defects in this region may underlie HS (Peters and Lux 1993).

Comparison of Erythrocyte and Brain Ankyrin

Ank2, the major isoform of ankyrin in the brain, is located on the plasma membranes of neurons and glial cells throughout the brain. It is structurally and biochemically similar to Ank1 at the protein level. However, the two proteins are unique products of separate genes, and have distinct binding properties. The sequence of ANK2 has been reported. In the protein 3 and spectrin binding domains, there is approximately 70% identity at the amino acid level with Ank1. Several regions, however, such as the C-terminal domain, completely diverge from the sequence of Ank1, probably serving unique functions to brain ankyrin.

Ank2, similar to Ank1, is subject to alternative splicing. Multiple isoforms arise as a result, with two major proteins, one of 220-kd (adult brain ankyrin) and another of 440-kd (fetal brain ankyrin), arising from a 9-kb or a 13-kb mRNA, respectively; these two major isoforms account for approximately 80% of human brain ankyrin. Studies in rats have shown maximal expression of the 440-kd isoform in the neonatal period, with subsequent targeting to unmyelinated axons and possibly dendrites (Peters and Lux 1993).

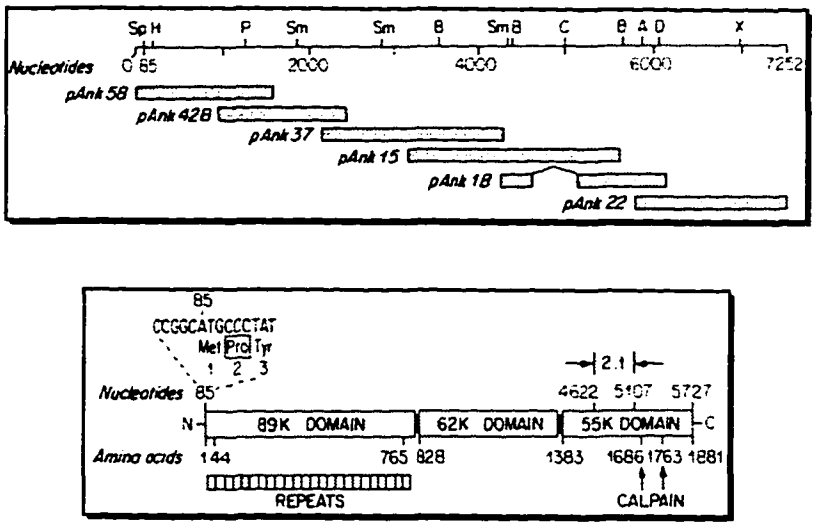

Figure 5 Organization of human erythrocyte ankyrin cDNA and protein. (Upper panel) Restriction map and clones. (Lower panel) Structure of the protein. Notice the three structural domains and the alternatively spliced "2.1" segment in the 55-kd (regulatory) domain. Other C-terminal isoforms, produced by alternative splicing, are not shown. (From Peters and Lux 1993)

Tissue and Developmental Stage-specific Alternative mRNA Processing

Two erythrocyte ankyrin transcripts were detected, first in our laboratory, by Northern blot analysis of human (9.0 and 7.2 kb) and mouse (9.0 and 7.5 kb) erythroid tissues (Lambert et al. 1990; Lux et al. 1990a). The ratio of the two transcripts depended on the stage of erythrocyte differentiation. In reticulocytes the 7.2/7.5 kb message predominated, whereas the 9.0-kb message was increased in earlier erythrocyte precursors in both species. When quantitated by laser densitometry, the 7.2:9.0 kb ratio was about 4 in human adult bone marrow and fetal liver erythroblasts, and greater than 6 in reticulocytes, indicating that the relative expression of the 9.0 kb message decreased as erythrocyte differentiation proceeds (Peters and Lux 1993). Of interest was the observation that the same ratio of the two ankyrin transcripts was substantially increased in the propositus in the HS pedigree under study, to a value of 10.83 ($p<0.009$) (Hanspal et al. 1991) .

The protein product of the 9.0 kb Ank1 transcript has not yet been defined, neither at the structural nor at the functional level. It has not even been clear whether a unique Ank1 isoform was produced, or whether the extra 2 kb of mRNA was untranslated sequence. However, it appeared reasonable to anticipate that a unique isoform existed, because extensive membrane remodeling has been known to occur during differentiation of the erythrocyte up to and including the reticulocyte stage and linker molecules with differing binding properties would be required to mediate such changes (Peters and Lux 1993).

Erythrocyte ANK1 expression is not limited to red blood cells. It has been reported to be highly expressed in the brain, especially the cerebellum, at a relatively late stage in the brain development, confined to a certain subset of neurons: Purkinje and granule cells in the cerebellum, most motor neurons of the spinal cord, and a small number of neurons in the forebrain. It has been, however, excluded from myelinated axons. Understanding the structure and the tissue- and developmental stage-specificity of ankyrin may have important

implications beyond the human blood. Particularly, in the proband, in whom, along with the severe hematological findings, there was a clear CNS involvement. Other sites of Ank1 expression have not yet been systematically analyzed (Peters and Lux 1993).

Ankyrin Deficiency

Our laboratory first reported the deficiency of spectrin and ankyrin in the propositus who had an atypical form of HS (Coetzer at al. 1988). Erythrocyte spectrin and ankyrin levels were half normal, apparently due to failure of ankyrin synthesis or to synthesis of an unstable molecule possibly subject to proteolytic degradation. Ankyrin mRNA concentrations were also half normal and newly synthesized ankyrin did not accumulate in the cytoplasm; only immunoreactive degradation products were detected. Additionally, ankyrin levels only reached half normal values in the membrane. In contrast, spectrin mRNA concentrations were normal, though only half of the synthesized spectrin attached to the membrane, presumably due to the lack of ankyrin sites (Hanspal et al. 1990).

Ankyrin defects have been known to occur in patients with HS, although their frequency remained unclear. This issue has been addressed by measuring RBC spectrin and ankyrin levels using radioimmunoassay, in cases of dominant HS kindreds. The values ranged from 40%-100% of the normal cellular levels of spectrin heterodimers and ankyrin molecules. Spectrin and ankyrin were both decreased in 75%-80% of kindreds studied (Savvides et al. 1991).

A combination of biochemical and molecular observations, together with cytogenetic and linkage studies, argues strongly that an ankyrin deficiency may be the primary defect in the majority of patients with dominant HS and that the spectrin deficiency may be secondary to the loss of ankyrin attachment sites. This evidence included reports of HS associated with either interstitial deletions of chromosome 8 or translocations of parts of this chromosome and chromosomes 3 and 12 (Bass et al. 1983; Chilcote et al. 1987; Lux et al. 1990b). The gene for ankyrin was mapped to chromosome 8p11.22 → 8p21.1 (Kitatani et al. 1988; Lux et al. 1990b). Additionally, in a large family the transmission of

HS has been linked to a polymorphism of the ankyrin gene (Costa et al. 1988; Costa et al. 1990). Furthermore, in one HS patient with interstitial deletion of chromosome 8, both the ankyrin DNA and the membrane-associated ankyrin were decreased (Lux et al. 1990b). Moreover, a dominantly inherited mutation in the regulatory domain of ankyrin was recently shown to be associated with HS in a pedigree of three generations (Jarolim et al. 1990). Finally, ankyrin deficiency has been described in patients with spectrin deficiency and in the propositus, the primary defect was shown to involve a decrease of ankyrin mRNA (Hanspal et al. 1987; Coetzer et al. 1988; Lambert et al. 1990; Hanspal et al. 1991; Savvides et al. 1991; Pekrun et al. 1993).

Molecular Basis of Spectrin and Ankyrin Deficiency in HS

Reticulocyte and cerebellum RNA of normal controls, as well as reticulocyte RNA of the proband, were used in Northern blot analysis with probes spanning the entire ankyrin cDNA. These studies revealed two major ankyrin transcripts, approximately 7.2 and 9.0 kb in size, whereas in human cerebellum a major transcript of 9.5 kb cross-hybridized with the erythrocyte ankyrin probes. Quantitation of the ratio between the two transcripts, by means of densitometry, revealed differences among a multitude of cells at different stages of development. In cultured erythroleukemia HEL cells the ratio was 0.7; it increased to 3.7 and 3.9 in purified fetal and adult erythroblasts respectively, and further increased to 6.15 in human reticulocytes. In the proband the ratio was increased to 10.83 ($p<0.009$).

Quantitative mRNA blots revealed an approximate 50% decrease in the propositus reticulocyte ankyrin mRNA when compared to normal controls, while there was no appreciable difference in the amount in several controls and HS proband α- and β-spectrin reticulocyte mRNAs (fig. 6). In addition, the higher molecular weight transcript was significantly less abundant in reticulocytes from the propositus (fig. 7), (Hanspal et al. 1991).

In control reticulocytes, α-spectrin was synthesized in excess of β-spectrin, yet the two chains were assembled in equimolar amounts forming a stable skeletal network, and the excess cytosolic spectrin was rapidly degraded. In the cytosol of the proband's reticulocytes, the synthesis of α-spectrin was identical to the control while β-spectrin synthesis was increased. On the proband's membrane, the two polypeptides were assembled stoichiometrically but their amounts were markedly reduced to about half the control values. The turnover of both cytosolic and membrane-associated spectrin appeared to be normal, as examined by a pulse-chase of control and propositus reticulocytes labeled with [^{35}S]-methionine and chased in medium containing unlabeled methionine. There was no detectable turnover of spectrin in the membrane; however, in the soluble fraction the amount of labeling initially increased after the onset of the chase and subsequently declined.

Despite normal (α-spectrin) or even increased (β-spectrin) synthesis, the assembly of the newly synthesized spectrin chains on the HS membrane was markedly reduced. The increase in β-spectrin synthesis might be related to a highly stimulated erythropoiesis. This was suggested by data showing that in erythroblasts isolated from the spleens of mice infected with the anemia-inducing strain of Friend virus (FVA cells) induced with erythropoietin, β-spectrin synthesis was selectively increased. Therefore, it was possible that the reticulocytes from the proband represented a similar system of stress erythropoiesis with a selective increase in the synthesis of β-spectrin (Hanspal et al. 1991).

Furthermore, the decrease in the assembly of newly synthesized spectrin on the membrane was likely to be related to abnormalities in the synthesis of ankyrin. In control reticulocytes, the amounts of ankyrin synthesized in the cytosol were in excess of those assembled on the membrane. In contrast, no intact ankyrin was detected in the cytosol of the proband's reticulocytes and only immunoreactive ankyrin degradation products were present. The newly synthesized ankyrin that was stably incorporated onto his membrane was markedly reduced to about half the control value. Incorporation of all other newly synthesized proteins in the proband's reticulocyte membranes, except spectrin and ankyrin,

appeared to be similar to control reticulocyte membrane, suggesting that the synthesis of other proteins was normal (Hanspal et al. 1991).

The findings of reduced ankyrin synthesis were consistent with the marked decrease of the ankyrin mRNA observed in the propositus. The normal chromosomal studies, as well as the absence of gross abnormalities on Southern blots using multiple restriction enzymes and two ankyrin probes, excluded the possibility of a deletion of a major fragment of the ankyrin gene. At this point, it remains to be proven whether the partial ankyrin mRNA deficiency is a result of mutation(s) leading to either a reduced expression or a reduced stability of the ankyrin mRNA (Hanspal et al. 1991).

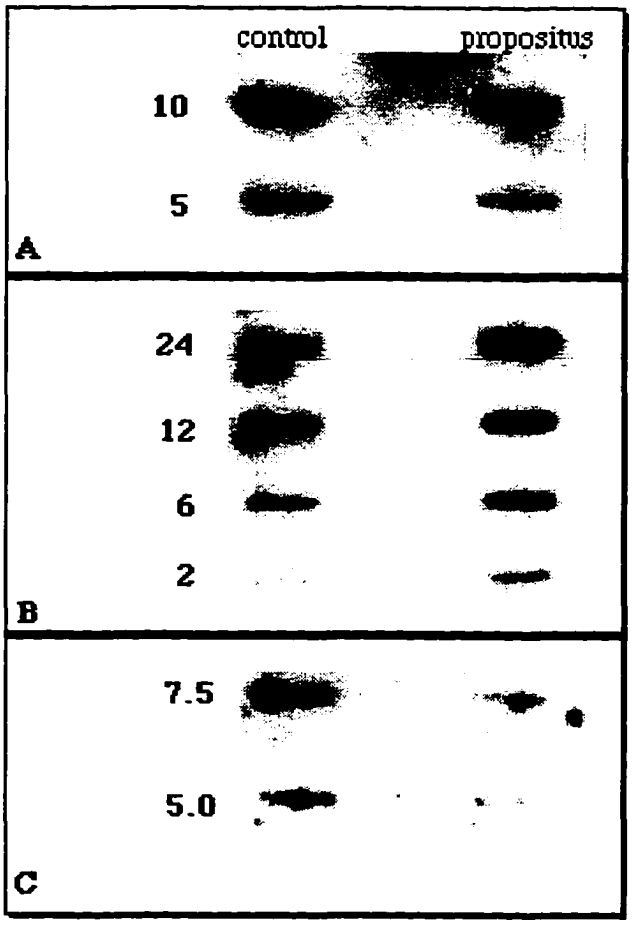

Figure 6 Quantitation of spectrin and ankyrin mRNAs in reticulocytes from the propositus and normal control. Poly $(A)^+$ RNA samples were applied on nitrocellulose filter by using a slot-blot apparatus. The filter was hybridized with (A) α-spectrin, (B) β-spectrin, and (C) ankyrin cDNA probes. While there were no significant differences in the amounts of control and propositus' α- and β-spectrin reticulocyte mRNAs, ankyrin mRNA was markedly reduced in reticulocytes from the propositus relative to the control sample. (From Hanspal et al. 1991)

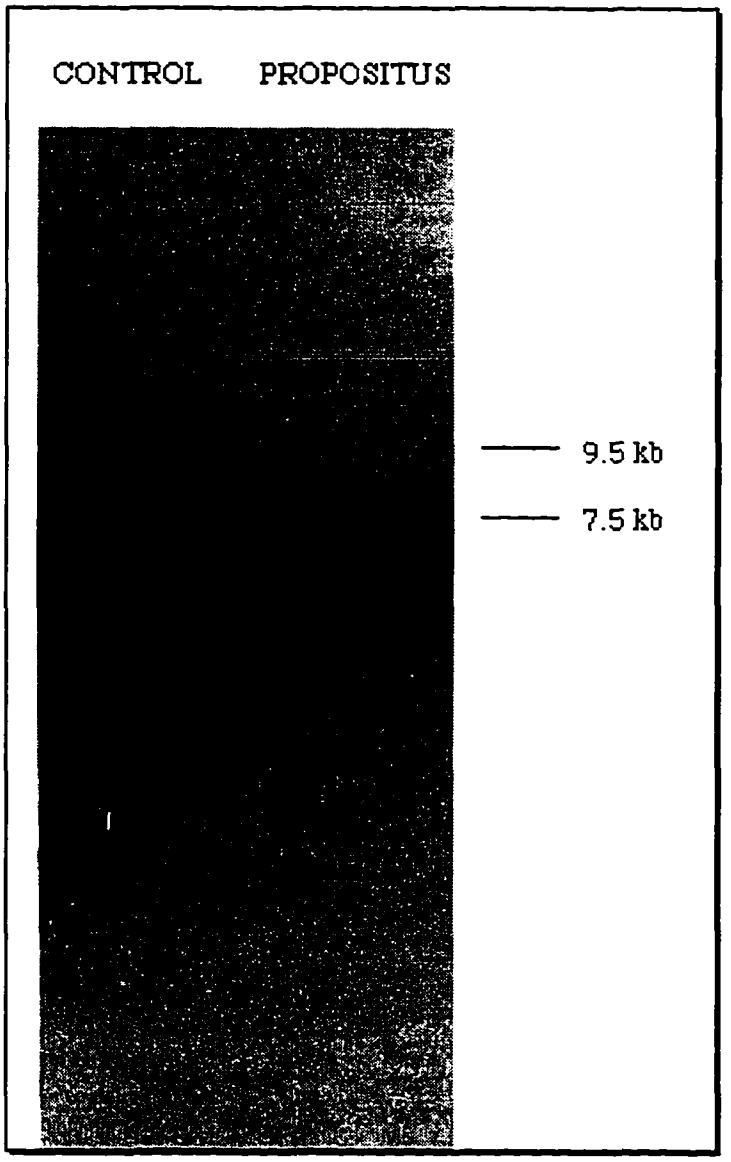

Figure 7 Representative Northern blot analysis of the propositus and control reticulocytes analyzed with an ankyrin cDNA probe. Fifteen micrograms each of poly $(A)^+$ RNA from the propositus and control reticulocytes were fractionated by formaldehyde agarose gel electrophoresis. After electrophoresis, RNAs were transferred to a nitrocellulose filter that was hybridized at 42°C with nick-translated ankyrin cDNA probe and washed at 65°C with low salt concentration solution. RNA ladder (Bethesda Research Laboratories, Gaithersburg, MD) served as molecular weight markers. Size markers were in kilobases. The film was developed after an 18-hour exposure at -70°C with intensifying screens. Note the two distinct ankyrin transcripts present in both the control and the propositus and that the amount of both transcripts were markedly reduced in reticulocytes from the propositus relative to the unrelated control sample. (From Hanspal et al., 1991)

Significance of the Proposed Work

Both spectrins and ankyrin play a key role in the formation of a stable skeleton in the erythrocyte membrane. This in turn determines its functional integrity. In the context of this project, the delineation of erythrocyte ankyrin domains, that upon mutation are associated with a disease state, will lead to a better understanding of the function of this protein. In addition, it will add to our knowledge about the functional integrity of the red cell membrane, in both health and disease states. Since ankyrin has been identified in other tissues as well, understanding the structure and the tissue- and developmental stage-specificity of this important protein may have significant impact beyond human blood. Eventually it may allow the design of more sophisticated strategies for those disorders where this protein malfunctions.

Specific Aims

1. To interpret the clinical data and the genetics of HS in this pedigree, in order to establish the inheritance pattern of HS in this family.

2. To confirm that the ratio of the erythrocyte ankyrin transcripts is abnormal in the propositus and to elucidate the nature of the abnormality by examining the possibility of alternative mRNA processing.

3. To confirm that the amount of mRNA is decreased in the propositus. This would have implications on the possible interaction of a null allele, as originally suggested, with a qualitative mutation from the other chromosome.

4. To screen for the presence of mutation(s) in the erythrocyte ankyrin cDNA in the propositus with a combined deficiency of spectrin and ankyrin associated with severe anisocytic HS and CNS abnormalities. Molecular mechanisms have not yet been identified.

5. To distinguish, if identified, between potentially disease-causing mutation(s) and benign polymorphisms also present in the general population that are not associated with a clinically significant phenotype.

RESEARCH DESIGN, MATERIALS, SUBJECTS, AND METHODS

Case Report

The propositus in this family of inherited spherocytosis of varying degrees, has recently expired at the age of 50. He was a white male who had had chronic hemolytic anemia since infancy, requiring transfusions. He had undergone a splenectomy at the age of 3 months, following which, his transfusion requirements became less frequent, his hemoglobin levels improved, but severe hemolytic anemia persisted. Since childhood he had had left hemiparesis with hemiatrophy, a chronic seizure disorder and psychomotor retardation. Long-term iron and transfusion therapy had resulted in hemochromatosis. When originally brought to medical attention, he had a hemoglobin concentration of 8-10 g/dl, a hematocrit of 20%-29%, a mean corpuscular volume (MCV) of 83-87 fl, a reticulocyte count of 4%-8%, a serum bilirubin concentration of 1.3-1.6 mg per deciliter (22-27 μmol per liter), and a serum lactate dehydrogenase concentration of 294-443 IU per ml. Osmotic fragility was considerably higher than normal, with initial hemolysis occurring in 0.85% NaCl and complete hemolysis in 0.65% (compared with 0.55% and 0.35% respectively, in controls). The peripheral blood smear revealed marked microspherocytosis. The patient's deceased father had severe anemia prior to his death at age 40 due to lung cancer (Coetzer et al. 1988). However, we were able to obtain medical records of the time of his induction in the army at the age of 19, at which time he had normal hemoglobin levels. Thus, it could not be determined whether the proband's father did or did not have a mild form of spherocytosis, because more sophisticated hematologic testing had not been performed at that time. Blood films or more detailed clinical information, such as osmotic fragility tests, were available neither at the VA hospital nor at the US military medical

records. His deceased mother had been hematologically normal. His parents' firstborn child was a girl who died of an unknown cause at the age of three months.

Routine Laboratory Evaluation

Most patients with HS either present with a mild anemia or with normal red cell counts and hematocrit and hemoglobin values, indicative of the very mild hemolytic rate, and with fully compensated hemolysis by the increased red cell production, as manifested by the presence of reticulocytosis. Despite the elevated percentage of reticulocytes, which normally have a larger volume compared to mature RBCs, the mean corpuscular volume of HS RBCs is frequently low normal or even somewhat decreased. This finding, in conjunction with the slight increase in the mean corpuscular hemoglobin concentration, reflects a mild cellular dehydration. However, some patients, particularly those with recessive forms of HS, are severely anemic, with hemoglobin levels greater or equal to 4 g/dl. In most patients there is evidence that indicates an accelerated red cell destruction. This is manifested by increased lactate dehydrogenase levels, unconjugated bilirubin and decreased haptoglobin, as well as reticulocytosis. These abnormalities may however be absent in milder forms of the disease.

Blood Film Evaluation

Spherocytes are readily identified by their characteristic shape on the peripheral blood film of patients with typical HS. They lack central pallor, their mean cell diameter is decreased, and they appear more heavily hemoglobinized. This finding reflects both the change in the red cell geometry and the increase in the cell density. In a three-dimensional view, some spherocytes have a stomatocytic appearance that is occasionally evident in the blood film. In a mild form of the disease, the appearance of the peripheral blood smear may be normal, because the loss of the surface area may be too small to be appreciated by such an evaluation. In such a case the cells look like "fat" disks rather than true spherocytes. Additional morphologic characteristics have been reported in patients with HS. Cells with irregular contour were present in the propositus with the combined deficiency of spectrin

and ankyrin. Pincered red cells were present in a subset of HS patients with band 3 deficit, while surface spiculation was manifested in a family with defective b-spectrin followed by abnormal interaction of spectrin and protein 4.1 (Palek and Jarolim 1995).

Osmotic Fragility

The osmotic fragility test measures the in vitro lysis of RBCs that are suspended in solutions of decreasing osmolarity. The red cell membrane is normally unstretchable and freely permeable to water. Therefore, the cell behaves as an ideal osmometer, in that it increases its volume when suspended in hypotonic solutions until a "critical hemolytic volume" is reached. The membrane then ruptures, and hemoglobin escapes in the supernatant through a single hole. Due to the loss of surface area material and the resulting surface area deficiency, the critical hemolytic volume in the case of spherocytes is substantially lower than in normal red cells. As a consequence, these cells hemolyze more than normal cells when suspended in hypotonic NaCl solutions. An elevated osmotic fragility is not unique to HS, though, and is also present in other conditions associated with spherocytosis on the peripheral blood film, such as immunohemolytic and Heinz body anemias. The dehydration that is evident in spherocytes (indicating decreased osmotic fragility in other conditions) appears to have no appreciable effect on HS osmotic fragility due to the markedly decreased cell surface area. In fact, the most dehydrated cells manifest the greatest increase in osmotic fragility. Osmotic gradient ectacytometry can precisely determine the relative contributions of cell dehydration and surface area deficiency. The osmotic fragility curve reveals uniformly increased osmotic fragility (fig. 8). Additionally, in nonsplenectomized HS subjects, a "tail" of the osmotic fragility curve may be present, reflecting a subset of particularly fragile red cells conditioned by the spleen. This subset disappears after splenectomy. In the case of mild HS, osmotic fragility may be normal and abnormalities may only be manifested after incubation that considerably enhances the loss of surface area; the sensitivity of incubated osmotic fragility may, however, be outweighed by the loss of its specificity.

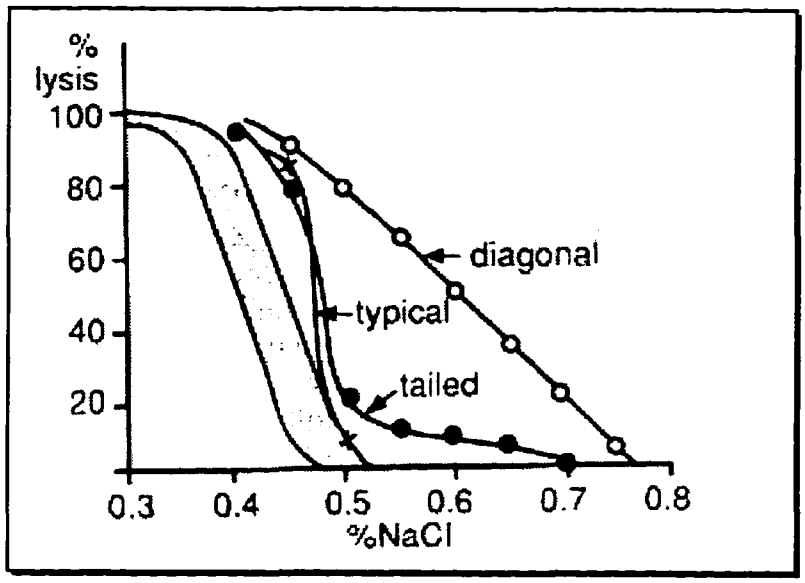

Figure 8 Characteristic osmotic fragility curves in hereditary spherocytosis. The typical curve of increased osmotic fragility is the most common finding. The tailed curve reveals a second population (tail) of very fragile erythrocytes. The diagonal curve is seen in patients with severe HS. (From Palek and Jarolim 1995)

Genomic DNA Isolation from Peripheral Blood

Ten ml of peripheral blood collected in EDTA-containing anticoagulant tubes from the propositus, other members of the affected family, and additional normal controls were used as starting material for the isolation of genomic DNA. The method of choice was based on the lysis of the erythrocytes using 1X lysis buffer for erythrocytes (1.55 M NH_4Cl, 0.1 M NH_4HCO_3, and 1 mM EDTA) until a completely white pellet of cells was obtained. To this white pellet of cells, 3 ml lysis buffer for the nuclear membranes was added; this contained 10 mM Tris-HCl, pH 8.2, 0.4 NaCl and 2 mM Na_2EDTA; additionally, proteinase K was included, to a final concentration of 100 mg/ml, and 200 ml of 10% SDS. The suspension was vortexed and incubated at 37°C overnight. The following day, after the addition of 6M NaCl and pure chilled ethanol, the DNA precipitated into solution within a few minutes and was literally "fished out" with the use of a glass hook. This was followed by washing of the DNA with 70% ethanol for approximately 10 min, air-drying of the pellet for 10-15 min and final resuspension of the DNA in the desired amount of dH_2O or TE buffer (Miller at al., 1988)

RNA Isolation and Northern Blot Analysis

The abundance of ankyrin transcripts in many tissues has been found to be extremely low and their size was large (Moon et al., 1985; Yoon et al., 1989; Prchal et al., 1990). Therefore, it has been very important that large amounts of the least degraded RNA be isolated for any kind of sophisticated analysis. The propositus, as well as additional subjects who were undergoing phlebotomies for hemochromatosis, served as the source of reticulocyte RNA. Typically, when it was available, 1 whole unit of peripheral blood (500 ml) was utilized as the starting material. Total RNA was isolated by the method using guanidine isothiocyanate as originally described by Chromczynski and Sacchi (1987).

For Northern blot analysis (Sambrook et al. 1989a) at least 40 µg of total RNA was utilized. Four and a half µl of the RNA sample was mixed with 2.0 µl 5X formaldehyde gel-running buffer, 3.5 µl formaldehyde and 10 µl formamide. The samples were

incubated at 65°C for 15 min before they were loaded onto a formaldehyde/1.4% agarose gel. Electrophoresis was performed at a constant 3-4 V/cm in 1X formaldehyde gel-running buffer until the bromophenol blue dye had migrated approximately 8 cm. At the end of the run, the RNA ladder-containing lane (Gibco, BRL) was excised from the gel and was visualized by staining with ethidium bromide (0.5 mg/ml) for 30-45 min. The marker was used to calculate the relative size of the messages analyzed. The RNA was then transferred overnight, as described by Sambrook et al. (Sambrook et al. 1989b), to a nylon membrane (GeneScreen Plus, DuPont/NEN) using the capillary elution method outlined by Sambrook et al. (Sambrook et al. 1989c). The filter was hybridized under stringent conditions with p(10-3'), a 1.5 kb EcoRI-SphI insert cloned into Bluescript (fig. 9). The probe was radiolabeled with [α-^{32}P] dCTP (3,000 Ci/mmol, Amersham, UK) utilizing the nick translation protocol (Boehringer Mannheim). The radiolabeled membrane was subsequently washed to remove the excess radioactivity and then analyzed by autoradiography; signal intensity was objectively quantitated using a phosphorimager.

5'-RACE

The protocol of 5' rapid amplification of cDNA ends, also known as 5'-RACE, was utilized to examine the 5' end of the ankyrin gene to gain insight into the issue of the substantially increased ratio of the two ankyrin transcripts present in the propositus (5'-AmpliFINDER RACE kit, CLONTECH, Clonetech Laboratories, Inc., Palo Alto, CA). The RACE protocol was first described by Frohman et al. and Belyavsky et al. and was later optimized by Edwards et al. (Frohman et al. 1988; Belyavsky et al. 1989; Edwards et al. 1991).

This a procedure for amplification of nucleic acid sequences from an mRNA template between a defined internal site and unknown sequence at the 5' end of the mRNA. The 5'-RACE can facilitate the isolation and characterization of 5' ends from low-copy messages. The general strategy was as follows (fig. 10). Approximately 5 µg of total reticulocyte RNA from the propositus and healthy individuals used as normal controls were

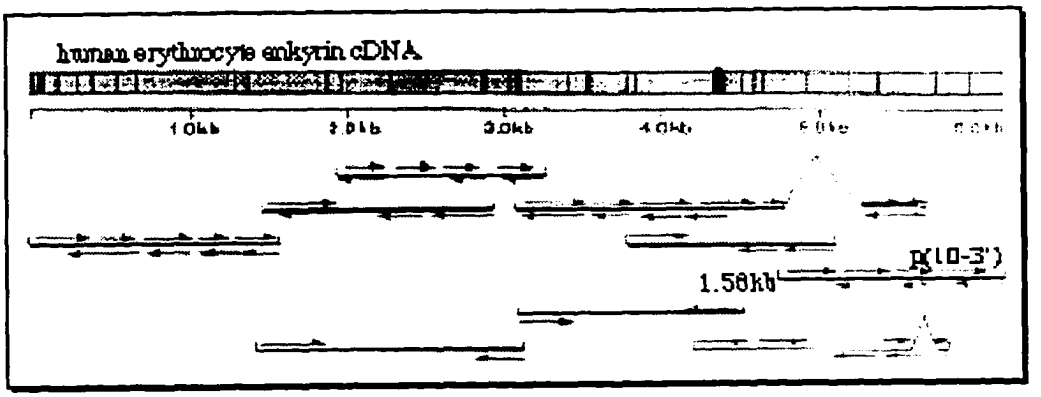

Figure 9 Organization of the ankyrin cDNA.(Lower panel) Alignment of clones in the composite cDNA sequence. Clone 10-3' (1.58 kb) was utilized as a probe in the Northern blot analysis. This cDNA clone was an *EcoR*I fragment cloned into pGM4. (From Lambert et al. 1990)

utilized as starting materials to prime first strand cDNA with the use of 10 μM of a gene-specific anti-sense primer (termed RT-1, reverse transcription primer), in a reaction also including reverse transcriptase buffer, 50 U RNase inhibitor, ultra pure dNTP mix (10 mM each) and 15 U AMV reverse transcriptase (all components were included in the kit). This permitted cDNA conversion of specific mRNA, or related families of mRNAs, and maximized the potential for complete extension to the 5' end of the message. Following cDNA synthesis, RNA was hydrolyzed with NaOH, and the first strand product was purified from unincorporated nucleotides and the excess gene-specific primer, and was precipitated using glycogen carrier, sodium acetate, and EtOH. Subsequently, 4 pmol of a specially designed single-stranded anchor oligonucleotide (fig. 11) was ligated to the 3'-end of the first-strand cDNA (typically, half the volume of the cDNA synthesis reaction). This was based on the ability of T4 RNA ligase to ligate single-stranded DNA molecules in the presence of hexamine cobalt chloride. Ligation was allowed to proceed for 16-20 h at room temperature (22°C). Following anchor ligation, a portion of the anchor-ligated cDNA (1 μl) was used as a template for PCR amplification, using a primer complementary to the anchor-primer and a second, nested gene-specific primer (TA-2) (table 4).

mRNA Preparation

Although it was possible to perform 5'-RACE using undegraded total RNA rather than purified mRNA as the starting material, more cycles of amplification were necessary when using total RNA. The use of additional amplification cycles was avoided as it led to accumulation of nonspecific products and an increase of artificial single nucleotide substitutions. To obtain the 5'-end of a gene like ankyrin, whose transcripts were both low in abundance and large in size, it was very important that the starting RNA material was of the best quality with least degradation.

cDNA Synthesis and Purification

cDNA quality and concentration was monitored by using the right combination of primers. If use of a gene-specific primer for the synthesis of cDNA was suboptimal, the

use of random hexamers was a useful alternative in priming cDNA and yielding material suitable for anchor ligation. Typically, 1 mM of primer was used to prime synthesis. An increase of this concentration led to carryover of excess primer in the anchor ligation reaction that competed with the anchor for ligation and was therefore avoided. A significant factor in obtaining efficient cDNA synthesis was disruption of secondary structure in the RNA template. In this respect, the temperature of the reverse transcriptase step was very important and was inhibitory for the formation of secondary structure. Relatively high temperatures of 42° to 46°C were used. cDNA purification was performed, primarily to avoid carryover of cDNA synthesis primers. For this purpose, the method based on the differential binding of the cDNA to a silica matrix was used (GENO-BIND^{TM}). Under high salt conditions, the cDNA preferentially bound to the matrix while the primers were effectively washed off. The cDNA was subsequently eluted in dH_2O, in a volume that ensured good recovery of the cDNA material from the silica matrix. A brief precipitation of the cDNA, using glycogen carrier and EtOH, was carried out prior to the anchor ligation.

Table 4

Oligonucleotide Primers Utilized in the 5'-RACE Protocol

---

anchor sequence(top strand):

EcoR I

3'NH_3- GGAGACTTCCAAGGTCTTAGCTATCACTTAAGCAC-P5'

5'-*CTGGTTCGGCCCA*- CCTCTGAAGGTTCCAGAATCGATAG-3' anchor primer sequence

reverse transcription primer (RT-1): 5'-CCTTCCTTAGAAGCCAGATGCAAGC-3'

ankyrin-specific primer (TA-2): 5'-GTTACCTGATCTTGCTGCTCTC-3'

Ligation

During the ligation of the oligonucleotide anchor to the cDNA preparation, the parameters monitored included the amount of cDNA and anchor to be ligated, as well as the temperature and duration of ligation. The amount of cDNA and anchor oligonucleotide had been optimized for the control that was included in the kit and the same amounts were used:

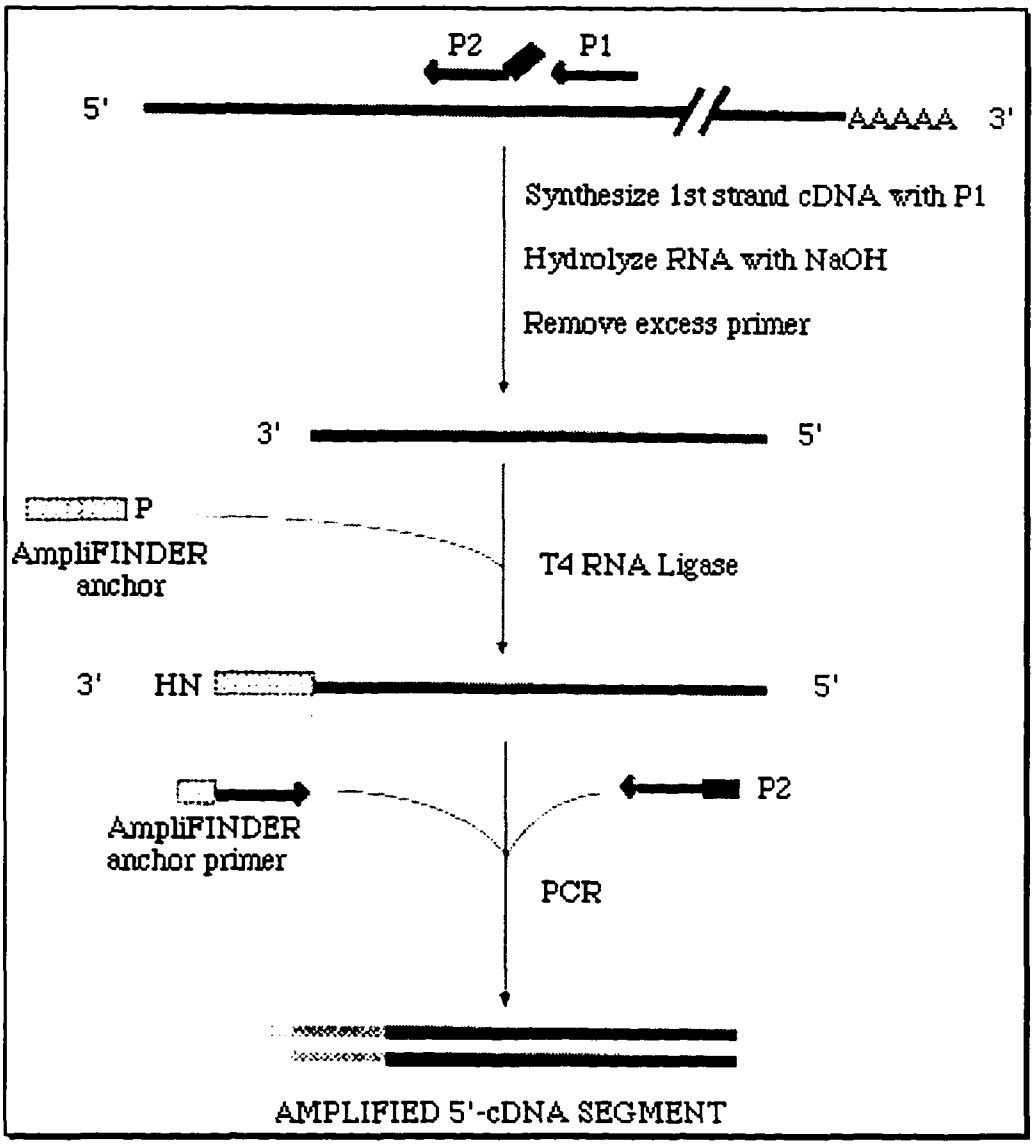

Figure 10 Amplification of the 5'-end of a gene using the 5'-AmpliFINDER RACE method. P1 and P2 are nested gene-specific primers. (From 5'-AmpliFINDER RACE kit protocol, CLONETECH, Clonetech Laboratories, Inc., Palo Alto, CA)

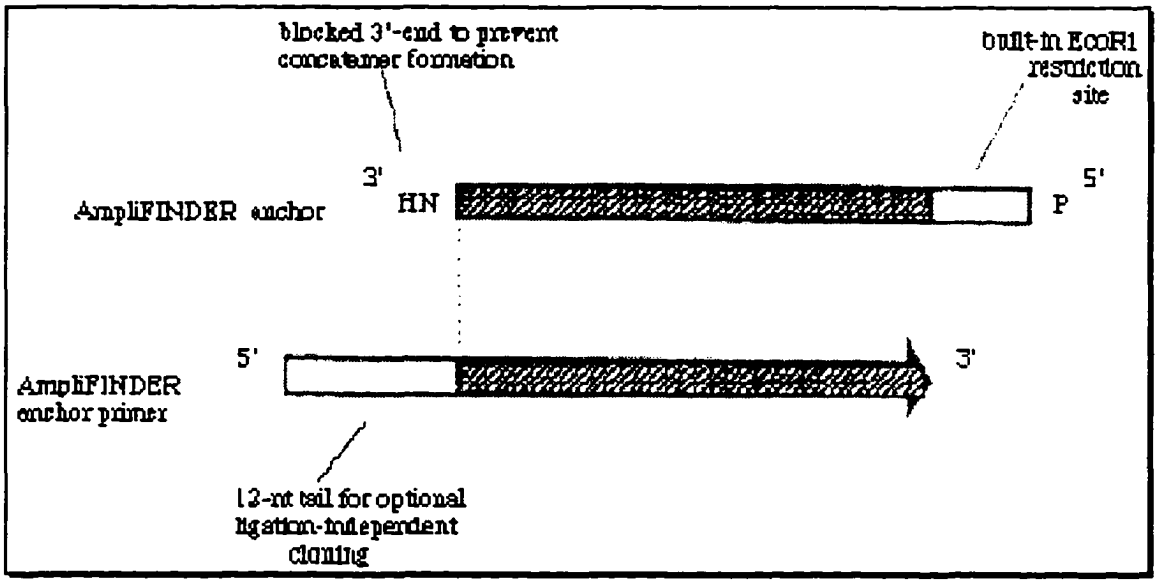

Figure 11 5'-AmpliFINDER anchor oligonucleotide, and anchor primer. The 3' end of the anchor has been modified with an amino to prevent concatamer formation; a phosphate group was added to the 5'-end; the extra 12-nucleotide sequence at the 5'-end of the anchor primer contains an *EcoR*I site and another sequence to facilitate optional ligation-independent cloning.

2.5 µl of the precipitated cDNA and 4 pmol of anchor oligonucleotide in a 10 µl ligation reaction. Other ligation components included the single-stranded ligation buffer and T4RNA ligase (20 U/µl). Ligation reactions were performed at room temperature (22°C) for 16-20 h. If the first ligation failed, there was always the remainder of the cDNA synthesis reaction for a repeat of the procedure.

PCR Amplification

Initially, the cycle parameters suggested in the protocol were used (annealing at 60°C was performed) and were subsequently adjusted for the gene-specific oligonucleotide primers used, to ensure optimal yield of the PCR products. For the anchor primer, optimal annealing was suggested at 55-65°C; above 65°C the efficiency was reported to be reduced. Usually, 1 µl of the ligation reaction was utilized for amplification, and it was not recommended that the amount was increased, in an attempt to avoid generation of a "smear" of products when electrophoresed on the agarose gel. A Mg^{++} of 2 mM, rather than the usual 1.5 mM, was used in the PCR reaction, because it improved the PCR product yield without resulting in nonspecific amplification.

Analysis of the Amplification Products

Following PCR amplification, products were analyzed by agarose gel electrophoresis, typically 1.8%, and ethidium bromide staining at 0.5 mg/ml. Band intensity and size distribution of the resulting PCR fragments usually depended on the specificity of the primers used for the cDNA synthesis and PCR, the complexity and relative abundance of the target cDNA, and the PCR conditions employed. Amplification products may vary from a single specific band to multiple discrete fragments to a broad diffuse smear.

Cloning and Analysis of the Amplification Products

The 5'-RACE cDNA products were subsequently cloned and sequenced. A broader band of PCR products was typically generated in most PCR reactions performed. The TA Cloning System (Invitrogen Corporation) was used for direct insertion of the PCR

products into the pCR^{TM}II plasmid vector (fig. 12). This system took advantage of the non-template dependent activity of the thermostable *Taq* polymerase (Gibco, BRL) used in PCR to add a single deoxyadenosine (A) to the 3'-end of the PCR products. Polymerases with extensive 3' to 5' exonuclease activity, such as *Vent* and *Pfu* DNA polymerases, should not be used because they do not generate 3' A-overhangs. These A-overhangs were used to insert the PCR product into the specifically designed vector that provided single 3' T-overhangs at the insertion site. The linearized vector supplied in the kit had single 3' deoxythymidine (T)-residues that allowed the PCR inserts to efficiently ligate with the vector.

The pCR^{TM}II vector (~50 ng) was ligated with 2 µl of the entire 5'-RACE PCR reaction, in a 10 µl reaction volume, also including 1X ligation buffer and T4 DNA ligase. The molar ratio of vector:PCR product used was 1:3. The ligation was performed at 15^{o}C for 10-12 h (overnight). One µl of each ligation reaction was then used to transform competent cells (with an efficiency of at least 1X10^{8} transformants/µg of supercoiled plasmid). The cells, which were very sensitive to temperature variations and mechanical lysis, were heat-shocked at 42^{o}C for exactly 30 sec. They were thawed on ice before any subsequent manipulations ensued. Different dilutions of the transformation were plated on LB-Agar plates containing ampicillin (50 mg/ml); X-gal was spread on top of the agar; IPTG was not required since One Shot^{TM} cells (INVαF') do not express the *lacI*^{q} repressor. Plates were incubated at 37^{o}C overnight. White colonies were picked for further analysis. For each insert subcloned, at least 10 white colonies were grown in 2-5 ml of LB broth containing ampicillin (50 mg/ml), and the plasmids were isolated for restriction mapping and nucleotide sequencing.

Subcloning of the PCR product(s), was followed by sequencing of the appropriate clones, utilizing the Sequenase^{TM} Version 2.0 DNA Sequencing Kit (United States Biochemicals), which used the chain-termination DNA sequencing method. This involved synthesis of a DNA strand by a DNA polymerase in vitro using a single-stranded DNA

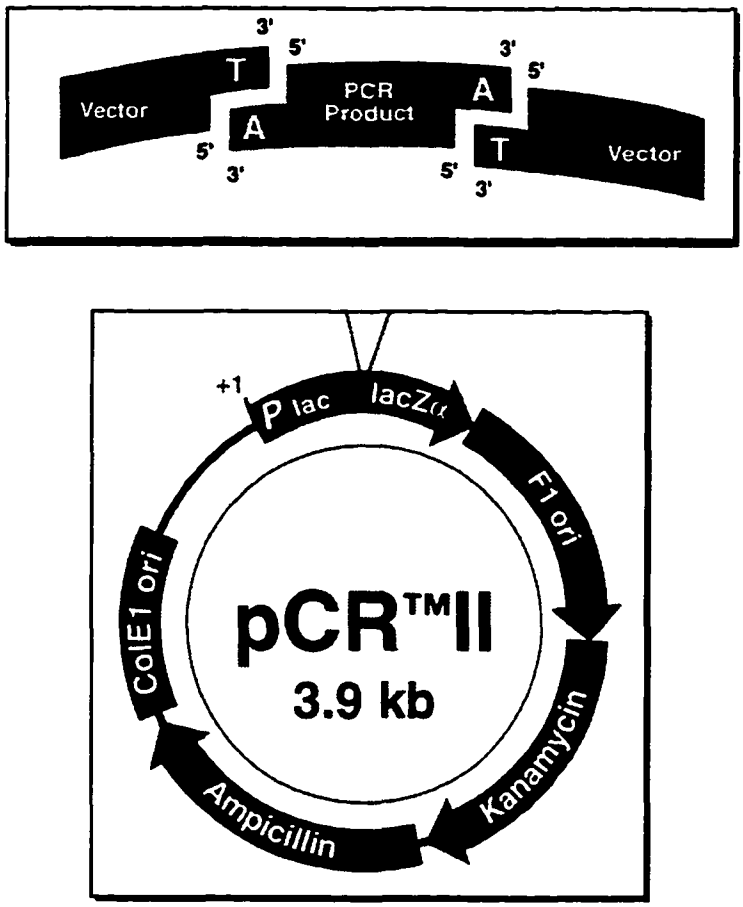

Figure 12 The concept of the TA cloning method, and the vector employed in the experimental process. *Taq* polymerase has a nontemplate-dependent activity which adds a single deoxyadenosine (A) to the 3'-ends of PCR products. The linearized vector has single 3' deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the vector. The lower panel illustrates the pCRII™ vector used in the TA cloning protocol. The vector has been modified at a unique *EcoR*I site during its preparation. The inserted PCR product is flanked on each side by *EcoR*I sites. (From TA cloning method protocol, Invitrogen Corporation)

template. Synthesis was initiated at only one site and was terminated by the incorporation of a nucleotide analog (e.g., ddNTPs) that would not support continued DNA elongation. When proper mixtures of dNTPs and one of the four ddNTPs were used, enzyme-catalyzed polymerization would be terminated in a fraction of the population of the chains at each site where the ddNTP could be incorporated. Four separate reactions, each with a different ddNTP, ensured the yield of complete sequence information. Including a labeled nucleotide (e.g., [^{35}S] dATP) in the reactions facilitated autoradiographic visualization of the labeled chains, after separation by high-resolution electrophoresis.

Approximately 5-7.5 µg of DNA was denatured in 0.2 M NaOH / 0.2 M EDTA by incubation at 37°C. The mixture was neutralized in 0.1 Vol 3M sodium acetate pH4.5-5.5. The DNA was then precipitated, washed, dried, and finally resuspended in dH_2O, sequenase buffer, and the sequencing primer (forward, reverse, and gene-specific primers have been used). DNA preparations of poor quality, along with primers and enzyme of low purity and quality, were the most common problems encountered with sequencing reactions. The primer annealed to the template by incubation at 65°C for 2 min and subsequent cooling to room temperature over a period of 30 min. To the annealed template-primer, a mixture of DTT, labeling mix, [α-^{35}S] dATP and sequenase enzyme was added and was incubated at room temperature for 2-5 min, before it was aliquoted to each one of the four preincubated at 37°C termination mixtures. The termination reaction proceeded for up to 30 min at 37°C, before it was stopped by the addition of stop solution. Before loading of the samples, the gel was prerun at 45 W for 20 min-1 h. Samples were denatured at 75-80°C for 3 min and were immediately loaded on the gel. Electrophoresis was performed at 45 W for approximately 3 h and the gel was then fixed and dried before autoradiography was performed.

Compressions

The largest problem encountered was elimination of compressions in the banding pattern. A multitude of methods have been proposed to eliminate compressions, such as

running the gel at the highest possible temperature. However, glass plates may crack at high temperatures and fuzzy bands may appear. Including up to 40% formamide in the sequencing gels (along with 7 M urea) eliminated very strong compression problems. Finally, certain nucleotide analogs were used if compression problems persisted. For this purpose, deoxyinosine 5'-triphosphate (dITP) at a 2-fold higher concentration than dGTP was used. In the termination reaction ddGTP was still used, but at a 5-fold lower concentration in order to obtain an average extension size the same as in the rest of the reactions. Additionally, the incorporation of 7-deaza-2'-deoxyguanosine 5'-triphosphate (7-deaza dGTP) has been suggested without increasing its concentration because it has the same efficiency of incorporation as dGTP.

Extension Lengths

Two parameters determining the average extension length of the synthesis reaction were the concentration of dNTPs in the labeling reaction and the dNTP: ddNTP ratio in the termination reaction mixtures. Under normal sequencing reactions, it was suggested (Ausubel et al. 1987) that a concentration of 0.2 mM for the three dNTPs be used in the labeling reaction, while a dNTP: ddNTP ratio of 10:1 in the termination reaction was recommended.

Required Levels of Labeled dATP

The preferred radiolabeled nucleotide was [α-^{35}S] dATP, over [α-^{32}P] dATP, because it provided higher resolution on sequencing gels, efficient incorporation by T7 DNA polymerase, and a lower risk to the operator. Under the conditions of the labeling reaction, the T7 DNA polymerase efficiently incorporated ~0.3 µl of 1,000-1,500 Ci/mmol of [α-^{35}S] dATP (Ausubel et al. 1987).

A helpful alternative way of analysis was sequencing of PCR products using the Sequenase PCR Product Sequencing Kit (USB). This offered a major advantage in that the possibility of identifying artificial changes that might be created by DNA polymerases was statistically much less likely to occur.

A highly specific PCR reaction was performed and the PCR products were then prepared for the sequencing reaction. No additional purification or separation steps were required. All gel purifications, sedimentations, or filtrations were eliminated by the use of two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase, used to treat the PCR products. Exonuclease I removed residual single-stranded primers and extraneous single-stranded DNA produced by the PCR. The Shrimp Alkaline Phosphatase removed from the PCR mix the remaining dNTPs, which would interfere with the labeling step of the sequencing step. Both enzymes were inactivated by heating to 80°C for 15 min.

Following enzymatic pretreatment the DNA was ready for sequencing with Sequenase DNA polymerase using standard techniques. The template was denatured and annealed to the gene-specific oligonucleotide primer using a heating and snap-cooling procedure. This was followed by the labeling and termination steps.

The amount of template DNA used in the reaction was very important. It was recommended that the minimum possible amount of template be used. Ideally, 0.2-0.5 pmol of template should be used. Good amplification reactions yield 10-20 ng of product per μl, but yields could vary tremendously depending on numerous factors.

Following the sequencing reaction, the quality of the gel electrophoresis was frequently the limiting factor of the extent of sequence information that could be derived from a single sequencing experiment. TBE gels should never be used with this kit, because the enzymes used contained sufficient glycerol to distort such gels. Instead, 20X Glycerol Tolerant Buffer was used.

3'-RACE

Next, evidence of alternative mRNA processing was sought at the 3'-end of the ankyrin gene that might explain the considerably higher ratio of the two ankyrin transcripts that was manifested in the propositus under study. Examples of 3'-terminal heterogeneity could include single or multiple poly(A) sites and multiple 3'-ends with an invariant splicing at the rest of the mRNA. In a similar way to 5'-RACE, the 3' end of the ankyrin gene was

analyzed using the protocol of 3'-RACE (fig. 13), (Frohman et al. 1988; Belyavsky et al. 1989; Edwards et al. 1991). To obtain cDNA 3' ends, this protocol required that RNA from the propositus and normal controls be reversely transcribed to create a cDNA strand. The primer that was utilized, a 57-base pair oligonucleotide, contained 17-dT residues designed to append to endogenous poly(A)tails (fig. 13); it also contained an adaptor sequence whose presence placed a unique sequence at the end of the cDNA. This sequence was sufficiently long to permit binding of two nested primers, P_{outer} (P_O) and P_{inner} (P_i). The first amplification was carried out using a gene specific primer (GSP1) and P_O. This amplified the cDNA of interest, as well as numerous other undesirable products. One way to minimize the problem of insufficient specificity in such a PCR amplification was to carry out an additional round of amplification using a set of nested primers. In this second round a small fraction of the first amplification reaction was re-amplified using a second, internal, gene-specific primer (GSP2) and P_i (table 5). The products were analyzed using 1% agarose gel electrophoresis and were then visualized with ethidium bromide staining at 0.5 mg/ml. The patterns of amplification were compared between the propositus and normal controls.

The effectiveness of this strategy depended on the assumption that nonspecific products from the first PCR reaction would not be amplified in the second reaction. The adapter sequence could be modified to include any kind of specific features that could facilitate further analysis and manipulation of the PCR products.

Reverse Transcription

While for most cases any standard procedure of reverse transcription might work well, special considerations had to be taken for a technique like 3'-RACE, to ensure as full of a cDNA extension as possible. The two important factors in this step were the RNA material and the oligonucleotide primer utilized to prime cDNA synthesis. As with the 5'-RACE protocol, very good quality RNA, free of reverse transcription-inhibiting agents, was required. Little RNA was essentially required, because even 100 ng of total RNA should contain approximately 100,000 copies of a rare message. Assuming a 70% conversion of

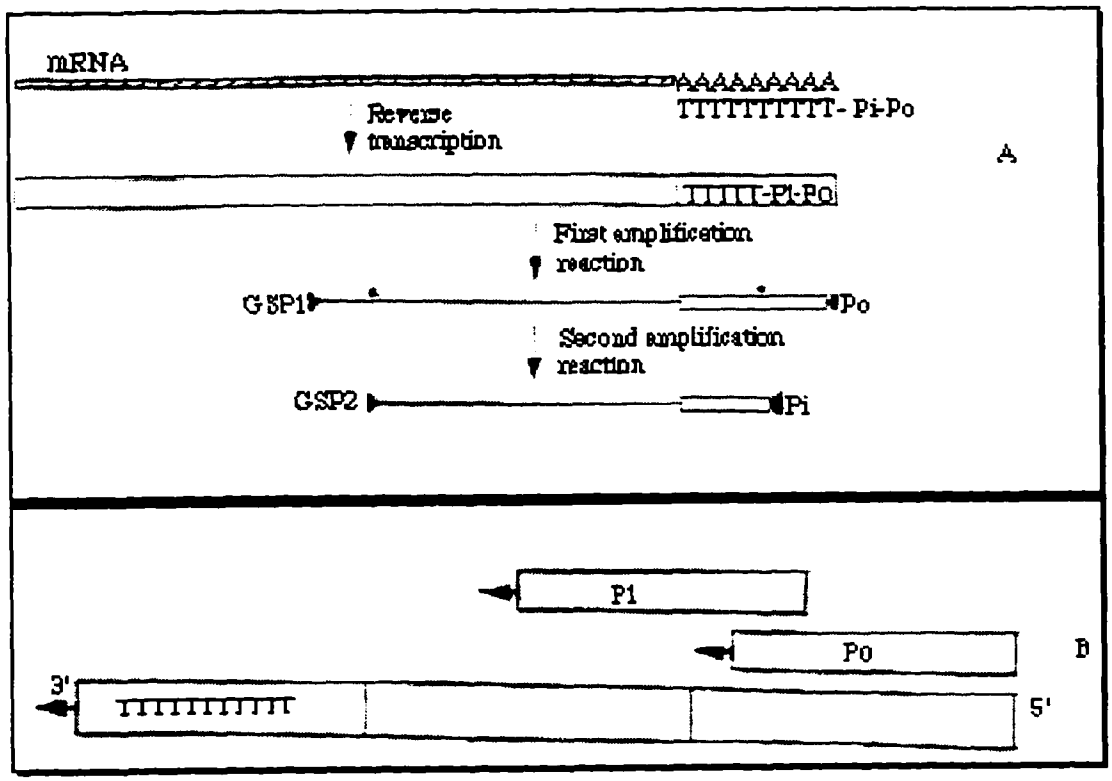

Figure 13 Schematic representation of the nested 3'-RACE end protocol. Initially, mRNA is transcribed using the $(dT)_{17}$-adapter primer. PCR amplification of the resulting cDNA is subsequently carried out using a GSP1 and the outer primer (P_o). A small fraction of this amplification product is then reamplified in a second PCR reaction using a nested GSP2 and the inner primer (P_i). (B).The adapter primer and the two nested PCR-primers used in the 3'-RACE protocol. The $(dT)_{17}$-adapter primer can be engineered to contain sequences that can facilitate subsequent analysis of the PCR products. The 17-mers P_i and P_o overlap by 6 nucleotides; this common region is not sufficient for annealing and extension under the PCR conditions employed. The sequence of the primers that were employed in the PCR reactions is shown in Table 5. (From Frohman and Martin 1989)

mRNA to cDNA, 5%-10% of that amount would be sufficient for amplification (Frohman et al. 1988). Since each of the cDNAs created contained a binding site for the adapter primer, a linear amplification of the entire cDNA pool was expected to occur at each cycle. If an excess amount of template was used, this background amplification would inhibit the exponential amplification of the intended cDNA. It was therefore important to limit the amount of the initial template utilized in the priming reaction.

Table 5

Oligonucleotide Primers Utilized in the 3'-RACE Protocol

$(dT)_{17}$-adaptor primer:	5'AAGGATCCGTCGACATCGATAATACGACTCACTATAAGGGA-TTTTTTTTTTTTTTTT 3'
Outer primer (P_o):	5' AAGGATCCGTCGACATC 3'
Inner primer (P_i):	5' GACATCGATAATACGAC 3'
ankyrin-GSP1:	5' GGACCCCATCTGAAAGG 3'
ankyrin-GSP2:	5' AGAGGAAGGAAAGGAGT 3'

Additionally, some problems could be encountered if excessive secondary structure was present. In such a case, performing the reverse transcription reaction at a higher temperature (up to 52°C) should be of help.

In regard to the $(dT)_{17}$-P_i-P_o oligonucleotide primer employed in the reverse transcription reaction, relatively small amounts were used (50 ng of primer per 20 µl reaction). Using a limiting amount of the primer increased the specificity during reverse transcription and decreased the probability of transcription being inhibited by binding of the excess primer at an undesirable site on the transcript. The adapter primer was used preferentially over random hexamer primers, since the latter may bind to a single RNA, creating either a prematurely terminated cDNA or one that was completely extended yet comprised of fragments that were not linked and would, therefore, become dissociated in the subsequent PCR amplification reactions.

Amplification

In general, a simple dilution of the reverse transcription reaction was used without phenol/chloroform extraction and precipitation prior to amplification. Sometimes, though, RNase treatment of the mRNA:cDNA hybrids could be important for efficient amplification. An extra extension time (~40 min) during the first round of PCR amplification, as was originally suggested, facilitated increased production of the specific product relative to background amplification, and increased the yield of long versus short cDNAs (Frohman et al. 1988). If the specific product could not be initially observed, treatment of the cDNA template prior to amplification was advisable (either with RNA hydrolysis or combination of RNAse H and RNAse A). Adding the *Taq* polymerase at 75°C, after a denaturation step at 95°C, eliminated the problem of primers being bound to undesirable sites and extending prior the PCR start.

The importance of choosing balanced primers was immense. While the sequence used for the adaptor primer was quite arbitrary, it had the same melting temperature (T_m) as the gene-specific primers employed. Annealing temperatures were safely set to maximize specificity and decrease background non-specific amplification and potential artifacts (Frohman et al. 1988).

The expected yield of the desired product relative to nonspecific amplified cDNA could vary tremendously depending on a variety of factors, primarily on the efficiency of the PCR amplification and the relative abundance of the transcript under study within the mRNA source. Since ankyrin transcripts have a very low abundance, it was decided that a second amplification, using a nested set of primers, would add an additional level of specificity to this step and would increase the likelihood of the exclusive amplification of the targeted cDNA. It was kept in mind that for an unequivocal confirmation of the specific amplification and further isolation of the candidate clones, Southern blot analysis and colony lift hybridization would be required. At each step, the amplification products were

visualized by 1% agarose gel electrophoresis followed by ethidium bromide staining at 0.5 mg/ml.

Amplification of the Entire Ankyrin cDNA

Our evidence did not indicate alternative mRNA processing occurring either at the 5'- or the 3'-end of the ankyrin gene that might explain the substantial increase in the ratio of the two ankyrin transcripts present in the propositus. This mandated the examination of the rest of the cDNA to search for any potential usage of alternative modes of exon splicing.

Standard PCR amplification was employed for this purpose. Nine pairs of oligonucleotide primers were designed, based on the published sequence of human erythrocyte ankyrin (ANK1) mRNA, that could effectively amplify the entire coding region of the gene (fig. 14), (Lambert et al. 1990; Lux et al. 1990a). These primers are outlined in Table 6. The PCR conditions that were employed each time varied, depending on the T_m of each individual set of primers as well as on the length of the target fragment amplified. Usually, 30 cycles were sufficient to permit effective amplification of the target fragments. Reversibly transcribed total reticulocyte RNA-derived cDNA from the propositus and from several normal controls were used side by side as starting material in the amplification reactions, and the respective patterns of amplification obtained were compared. The PCR products were visualized on 0.8%-1.2% agarose gels stained at 0.5 mg/ml ethidium bromide. Table 7 summarizes the PCR cycling conditions employed in these reactions.

Single Strand Conformational Polymorphism Analysis

Scanning for the presence of mutations in the propositus, in comparison with a number of normal controls, was performed utilizing the technique of single strand conformational polymorphism (SSCP) analysis. SSCP was based on the principle that single-stranded DNA molecules take on specific sequence-based secondary structures under non-denaturing conditions (Orita et al. 1989a; Orita et al. 1989b; Hayashi 1991; Cotton 1993; Sheffield et al. 1993). Under nondenaturing conditions, single-stranded DNA

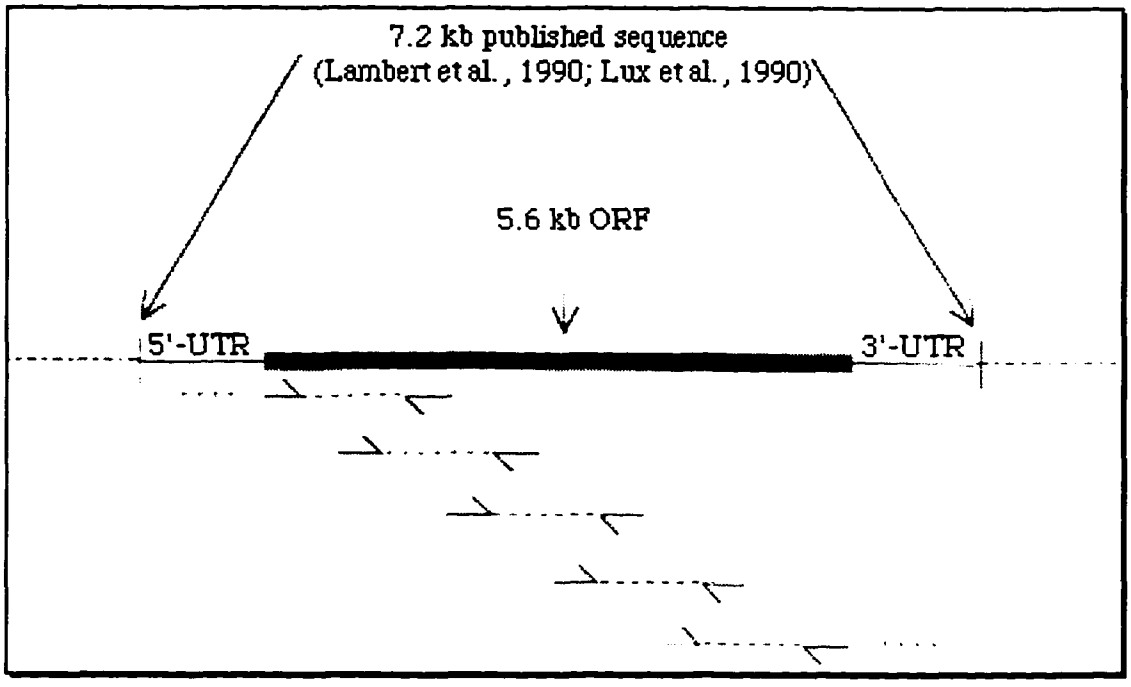

Figure 14 Schematic representation of the human erythrocyte ankyrin cDNA (ANK1), and the overlapping oligonucleotide primers spanning the entire sequence. These oligonucleotide primers were designed based on the published cDNA sequence of ANK1 and are summarized in Table 6. (From Lambert et al. 1990; Lux et al. 1990a)

Table 6

Primers Used in the Amplification of the Ankyrin cDNA and SSCP Protocol.

Primer pairs	Primer sequences	PCR product
1FOR *1REV*	5'CCCAGCTGCTCCTCCTCAAG3' 5'TGGTTTTGGCTTGGATTGGT3'	967 bp
2FOR *2REV*	5'CACCACAGAACGGCATCACG3' 5'CCACGTGCAGAGGGGTAAAT3'	924 bp
3FOR *3REV*	5'TGCATGACCAAGAAAGGATT3' 5'GCAGCTCCTTCTCTTCATCA3'	940 bp
4FOR *4REV*	5'CGGAAGATGAAGGGGAAGAA3' 5'GGAAGTCGGTGGTGATGATT3'	729 bp
5FOR *5REV*	5'CGAATCATCACCACCGACTT3' 5'TTCGGCTCCACTTCCCTTAG3'	963 bp
6FOR *6REV*	5'AGTACGAGGACACCCAGCAC3' 5'CGCGTCATCCTGCCTCTTAG3'	935 bp
7FOR *7REV*	5'TATCGATTTGCTTGAACAGG3' 5'CCTGCGCTTGTTTTCTATCC3'	1,002 bp
8FOR *8REV*	5'GGAACCAGGACACAAAAGCA3' 5'GCTCTCCGCCTCCGACTCTA3'	1,185 bp
9FOR *9REV*	5' GGACCCCATCTGAAAGG 3' 5'GATGGCACCGAGGCGAGGAA3'	579 bp

Note: The 5' end of *1FOR* is at nt 25, and the 3' end of *9REV* is at nt 7,176, amplifying 7,150 bp of the ankyrin cDNA. (Lambert et al. 1990; Lux et al. 1990a).

Table 7

Reaction Conditions in the PCR Amplification of the Ankyrin cDNA.

Primer Set	cDNA amount[a] (µl)	T_m (°C)	*Taq* pol (units/reaction)[b]	# cycles
1FOR/1REV	5	65	1.5	35
2FOR /2REV	3	66	2	35
3FOR /3REV	2.5	67	2	35
4FOR /4REV	2.5	66	2	35
5FOR /5REV	1	60	1.5	30
6FOR /6REV	1	68	1.5	30
7FOR /7REV	1	60	1.75	30
8FOR /8REV	1.5	63	1.75	30
9FOR /9REV	5	63	1.5	35
6FOR /7REV	5	60	5	35

[a] out of 20µl reverse transcription reaction; per 50 µl PCR reaction.
[b] *Taq* polymerase from Gibco, BRL, 5u/µl concentration.

had a folded conformation stabilized by intramolecular interactions. As a consequence, its conformation as well as its mobility depended on its sequence (Orita et al. 1989a). Molecules differing by as little as one base substitution formed different conformers and migrated differently in a nondenaturing polyacrylamide gel. Thus, in SSCP analysis, a mutated sequence was detected as a change in mobility in polyacrylamide gel electrophoresis caused by an alteration in its folded structure. Because of its high resolving power, polyacrylamide gel electrophoresis (PAGE) had the capacity to distinguish most conformational changes caused by sequence differences such as one base substitution in a 200-300 base pair fragment. The shift of electrophoretic mobility induced by a mutation could not possibly be predicted.

The important steps involved in this methodology included: total reticulocyte RNA isolation, reverse transcription for cDNA, PCR amplification of the cDNAs using the nine pairs of oligonucleotide primers outlined in Table 5, PCR product purification, purified PCR product restriction endonuclease digestion, finally followed by denaturation and electrophoretic analysis of the fragments of interest under non-denaturing conditions and visualization of the results by silver staining. The propositus was analyzed along with several normal controls. Further analysis of suspected mutations manifested as mobility shifts included subcloning and sequencing, or sequencing of the PCR products alone.

cDNA Synthesis

Total reticulocyte RNA (approximately 1-2 μg), from the propositus and normal controls was reversely transcribed in a 20 μl reaction volume containing 1X First Strand buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 5 mM DTT, 0.75 mM each dNTP (Boehringer Mannheim), 1 mg of random hexanucleotide primers (Pharmacia), 10 U of RNasin (Promega), and 200 U of Superscript II M-MLV H^-reverse transcriptase (Gibco BRL).The RNA and the random primers were heated to 70°C for 10 min prior to addition of the rest of the reverse transcription components. The reaction was allowed to proceed for 75 min at 42°C, followed by reverse transcriptase inactivation by incubation at 70°C for 5

min. After addition of all the components and of the reverse transcriptase, the reaction was incubated at room temperature for 10 min, facilitating a higher yield of better quality cDNA. An aliquot of the cDNA reaction was always PCR amplified using a set of actin control primers, in order to monitor the quality and relative quantity of the cDNA and to provide an indication of the amount that would be used in the ankyrin-specific amplifications

PCR Amplification

Following cDNA synthesis, the entire cDNA region of erythrocyte ankyrin was PCR amplified with nine pairs of oligonucleotide primers (Oligos Etc., Inc., and UAB Core Facility) that were designed (using Oligo 4.0 primer design program) based on the published sequence of human erythrocyte ANK1 (Lambert et al. 1990; Lux et al. 1990a). The nine sets of primers are listed in Table 6. Conditions of amplification varied with each pair of primers, but typically included an initial denaturation step of 10 min at 95°C, followed by 30-35 cycles of a 3-step PCR, and a final extension step of 10 min at 72°C, in a GeneAmp PCR thermalcycler 9600 (Perkin Elmer Cetus). One hundred µl reaction volumes were used containing 1X PCR buffer (20 mM Tris-HCl pH8.4, 50 mM KCl), 2 mM $MgCl_2$, 0.2 mM each dNTP (Boehringer Mannheim), 10 pmol of each primer and 2.5 U of *Taq* polymerase (Gibco BRL). PCR products were visualized by gel electrophoresis on a 1% Agarose gel stained with ethidium bromide at 0.5 mg/ml.

PCR Product Purification/Restriction Endonuclease Digestion/PAGE

Following the PCR amplification reactions the products were electrophoresed in TAE-containing 1% agarose gels. The bands were excised from the gel and subsequently purified using the QIAEX II Gel Extraction kit (QIAGEN kit). One-to-two mg of DNA was subsequently digested with the appropriate restriction endonucleases in a 10 ml reaction volume. The endonucleases were chosen based on the published sequence of human erythrocyte ANK1 using the MacDNASIS Pro v3.0 program, aiming at the generation of fragments of optimal size (150-250-bp) for SSCP analysis (Lambert et al. 1990; Lux et al. 1990a). Digests were performed at 37°C for at least 2 h to ensure the complete digestion of

the target cDNA. The presence of incompletely digested material would complicate the interpretation of the results because of the presence of numerous conformers. Digestion products were visualized by gel electrophoresis on a 1% Agarose gel stained with ethidium bromide. An aliquot of each digest (usually 1 ml) diluted with 9 ml dH_2O and 10 ml of formamide-loading buffer (95% formamide, 5 mmol/L, 0.1% Bromophenol Blue, 0.1% Xylene Cyanol) was then denatured (for 8 min at 95°C) and rapidly snap-cooled on ice. All 20 µl of the reaction was loaded on a nondenaturing PAGE. The concentration of the gel depended on the size of the fragments that were analyzed each time and was typically 6%-12%; it also contained 5% glycerol and 0.5X TBE. Electrophoresis was performed at 50 W constant power for 3 h at a constant temperature of 14°C. Gels were then fixed and silver stained (Silver Stain kit for Polyacrylamide gels, SIGMA) for visualization of the results.

Putative mutations in the coding region of ankyrin were manifested as band mobility shifts. The samples that demonstrated the presence of a possible mutation by SSCP were subjected to subcloning and sequence analysis. Sequencing was performed in both the sense and anti-sense orientation. Table 8 summarizes the oligonucleotide primers that were utilized in the sequencing reactions.

Sensitivity of SSCP

SSCP analysis has been used with great success for the identification of mutations and polymorphisms. The sensitivity of SSCP had been reported to be high (Hayashi 1991; Michaud et al. 1992). When different sized products were analyzed by SSCP, a striking relationship between sensitivity of mutation detection and PCR fragment size had been observed, with the highest sensitivity noted for most fragments of approximately 200 bp or less in length. In a study by Sheffield and co-workers, 70% of single base substitutions were detected in two globin fragments of 212 bp in length (Sheffield et al. 1993). The same study reported a significant decrease in the sensitivity of the technique (only 1 of 29 mutations was detected in a fragment of 600 bp in length). Substantially greater sensitivity was noticed with molecules shorter than 200 bp in length (96% and 97% mouse globin

mutation detection in two 155 bp fragments). The study suggested that there was a lower limit of size, presumably due to the constraints placed on the ability of a relatively small fragment to form a stable secondary structure that could be analyzed by SSCP.

Additional factors that could theoretically affect the sensitivity of SSCP for detecting single base substitutions included the position of the base substitution within the fragment, the nature of the base substitution, and the sequence composition of the fragment analyzed. All of these factors would be expected to influence the secondary structure of the molecule, although the exact role of each would be difficult to evaluate. Mutations not detected by SSCP were likely to be interspersed within the sequence, rather than within a contiguous stretch of bases. The type of mutation did not seem to play a significant role in determining whether the mutation could be detected by SSCP. Both transitions (81%) and transversions (76%) had been detected at similar overall frequencies. Interestingly, G-to-T transversions were detected at a substantially lower rate (57%). However, since the complimentary C-to-A transversion was more efficiently detected (82%), it was not determined whether the low frequency of the G-to-T change was a generalized phenomenon.

Table 8

Oligonucleotide Primers Utilized in the Sequencing Reactions.

primer	primer sequence (5'→3')	5'-nt position	3'-nt position
2for	CACCACAGAACGGCATCACG	788	807
2for$_B$	ACCACCTGACCCCACTCCAC	1,094	1,113
2for$_C$	GGAGACCACCTCGACTGTGT	1,027	1,046
2for$_D$	GCACATTGCGGCTCACTACG	714	733
2for$_I$(wt)	CTGCTGAAGACGGGAGCCT	1,252	1,270
2for$_{II}$(wt)	CCACGTGGCCTCCTTCA	1,308	1,324
2rev	CCACGTGCAGAGGGGTAAAT	1,711	1,692
2rev$_B$	TGGCATTGACTTTGGCTTTG	1,480	1,461
2rev$_C$	CACTTTCACGTTGGAGACGT	1,395	1,376
4for	CGGAAGATGAAGGGGAAGAA	2,534	2,553
4for$_B$	AGCAGGAGCAGGCATCTAAA	2,711	2,730
4rev	GGAAGTCGGTGGTGATGATT	3,262	3,243
4ev$_B$	ACGATTACAGGGCTCAGGAA	3,056	3,037

The actual base position of the substitution may be more important than the precise nucleotide substitution in determining mobility shift. Different mutations at the same position usually gave identical results; either they both did not result in a shifted band or they both resulted in nearly identical shifts, showing that, at least in certain instances, SSCP would be limited by the inability to distinguish between different base substitutions at the same nucleotide position. The extent of this limitation had not been determined yet.

Additionally, modification of the sequence flanking mutations could result in altered sensitivity of mutation detection by this assay. It was hypothesized that when the sequence flanking the substitutions could participate in the formation of secondary structure with the region of mutation, the mutation would then be detected. Of interest was the observation that the sensitivity of mutation detection was frequently greater for one strand of the DNA sequence rather than the complimentary one. Finally, SSCP sensitivity had been increased by analysis of the same fragment with different gel conditions (Orita et al. 1989b). It should be born in mind nevertheless, that a single strand may form a different number of conformers under different gel conditions (Hayashi 1991; Sheffield et al. 1993).

Lately quite a few improvements have been described. For example, the adaptation of precast gels and the application of silver staining has eliminated the use of radioactivity, and has improved resolution (Cotton 1993). Mutations could be most effectively screened for in fragments of several hundred base-pairs long. For example, a DNA fragment 900 bp in length was digested with several frequent-cutting restriction enzymes and was subjected to SSCP analysis (Iwahana et al. 1992). This approach both increased the length screened and improved the chances of mutations being detected due to different fragment contexts for the mutation. The most marked modification of the technique has been the transfer of analysis to RNA, the rationale being that RNA can assume more elaborate and greater numbers of conformational forms that appear to be sensitive to single-base substitutions (the frequency of mutation detection in a fragment of 183 bp was reported to be 93% with the use of RNA SSCP, compared to 83% of DNA SSCP) (Cotton 1993).

Silver Staining

The use of silver staining of acrylamide gels in SSCP analysis has been a highly sensitive visualization technique that allowed detection of polypeptides of as little as 0.1-1.0 ng in a single band (Ausubel et al. 1987b). The process relied on the differential reduction of silver ions bound to various chemical groups (e.g., sulfhydryl and carboxyl moieties) of polypeptides, which has been the basis for photographic processes. The most common methods fall into two main classes: those that use ammoniacal silver solutions and those that utilize silver nitrate. Highly sensitive photochemical silver staining techniques permit the detection of polypeptides in gels at about 100- to 1,000-fold higher sensitivity than staining with Coomassie Brilliant Blue R250. The use of silver nitrate solutions has made the technique easy to work with and, in contrast to ammoniacal solutions, has eliminated the generation of hazardous and explosive by-products.

The high sensitivity of the silver staining technique rendered it susceptible to impurities and staining artifacts. In this respect the concentration of PCR fragment subjected to analysis was closely monitored to avoid artifacts that might have complicated the interpretation of the results. It was imperative that the polyacrylamide gels and all staining solutions be prepared from high quality reagents in order to avoid any staining artifacts. Especially important appeared to be the use of high quality reagents. It was decided that a commercial silver staining kit, the Silver Stain Kit for Polyacrylamide Gels from Sigma, was to be used, since silver staining was infrequently performed in our laboratory.

The most clear-cut advantage of SSCP as used for DNA was the apparent simplicity of the method. Use without a label was also a significant advantage. However, this was offset by the most serious disadvantage of the method, which was the lack of 100% detection of potential mutations. It has been suggested that if an application could tolerate around 80% detection, then SSCP was the procedure of choice. Such applications included

detection of polymorphisms in a family for use in disease gene linkage studies, or finding mutations in a specific gene, which had been one of our main aims.

Completion of SSCP and identification of suspected mutations manifested as mobility shifts was followed by analysis of the cDNA fragments that demonstrated the shifts. Analysis included subcloning into the pCRII^{TM} vector using the TA cloning protocol. The candidate clones containing the inserts of interest were analyzed by SSCP using the same restriction endonucleases that had revealed the original mobility shifts; clones that migrated similarly when compared to the digests that had originally revealed the change in the migration pattern in the propositus were chosen for nucleotide sequencing of their inserts utilizing the Sequenase^{TM} Version 2.0 DNA Sequencing Kit (United States Biochemicals). One of the normally migrating clones that was derived from subcloning cDNA material from a normal control was chosen to be used as a normal standard and was further sequenced in the same way as above. Alternatively, sequencing of the PCR fragments of interest was pursued as a helpful option, using the Sequenase PCR Product Sequencing Kit (United States Biochemicals), as previously described, the rationale being that such an approach would make the possibility of identifying artificial changes created by DNA polymerases statistically less likely to occur. Sequencing was performed in both the sense and anti-sense orientations; additionally, the analog 7-deaza-dGTP was incorporated, in order to eliminate compression problems that were encountered.

Allele-Specific PCR (ASPCR)

This recently described technique is a highly specific, rapid, reproducible, and inexpensive assay for the detection of already identified specific single-base mutations or polymorphisms (fig. 15), (Wu et al. 1989; Sommer et al. 1992; Xu and Hall 1994; Liu et al. 1994). It was based upon the observation that a mismatch at the 3' end of a primer used in a PCR amplification reaction would prevent effective amplification. Only DNA polymerases without proofreading activity should be used for the purposes of such an assay. Conceptually, primers that matched the template sequence perfectly would

consistently generate a PCR product. On the other hand, primers with a mismatch at the 3' end of the primer (the allele-specific primer) would not generate a PCR product. ASPCR could be used for population screening, haplotype analysis, patient screening and carrier testing. Multiplex ASPCR could also be employed for screening analysis at more than one site per PCR reaction (Sommer et al. 1992).

A number of parameters could be optimized to obtain the desired level of specificity (Sommer et al. 1992). These parameters included:

(1) Oligonucleotide concentration: decreasing the oligonucleotide concentration to 0.05 mM has been reported to increase specificity. However, below 0.025 mM the signal of amplification generally became weak.

(2) Magnesium concentration: Specificity was be increased by a low magnesium concentration, sometimes lower than 1.5 mM. Adding EDTA, could be a simple way of decreasing the "effective" magnesium concentration without having to resort to making a separate PCR buffer/salt stock solution. Likewise, a magnesium concentration higher than 4.5 mM can lead to the generation of non-interfering spurious amplification products that could be used as internal controls.

(3) Nonspecific oligonucleotide of the primer pair: Occasionally, when a pair of primers could not achieve specificity, its replacement with a new oligonucleotide at a new location should accomplish the desired level of specificity. It was advisable that the nonspecific oligonucleotide be replaced with the new specific one, so that it amplified a smaller fragment of DNA. In general, the optimal sizes of products were between 300-600 bases. This was not always possible. It has been reported that fragments varying in size from 200-2,700 bases in length have been specifically amplified.

(4) DNA concentration: Specificity was increased by use of a 10-fold dilution of the standard genomic DNA concentration (i.e., 1 ng/ml) without leading to an inadequate amplification signal. Dilution of the template could additionally increase sensitivity and avoid problems arising from contamination of the DNA with any PCR inhibitors.

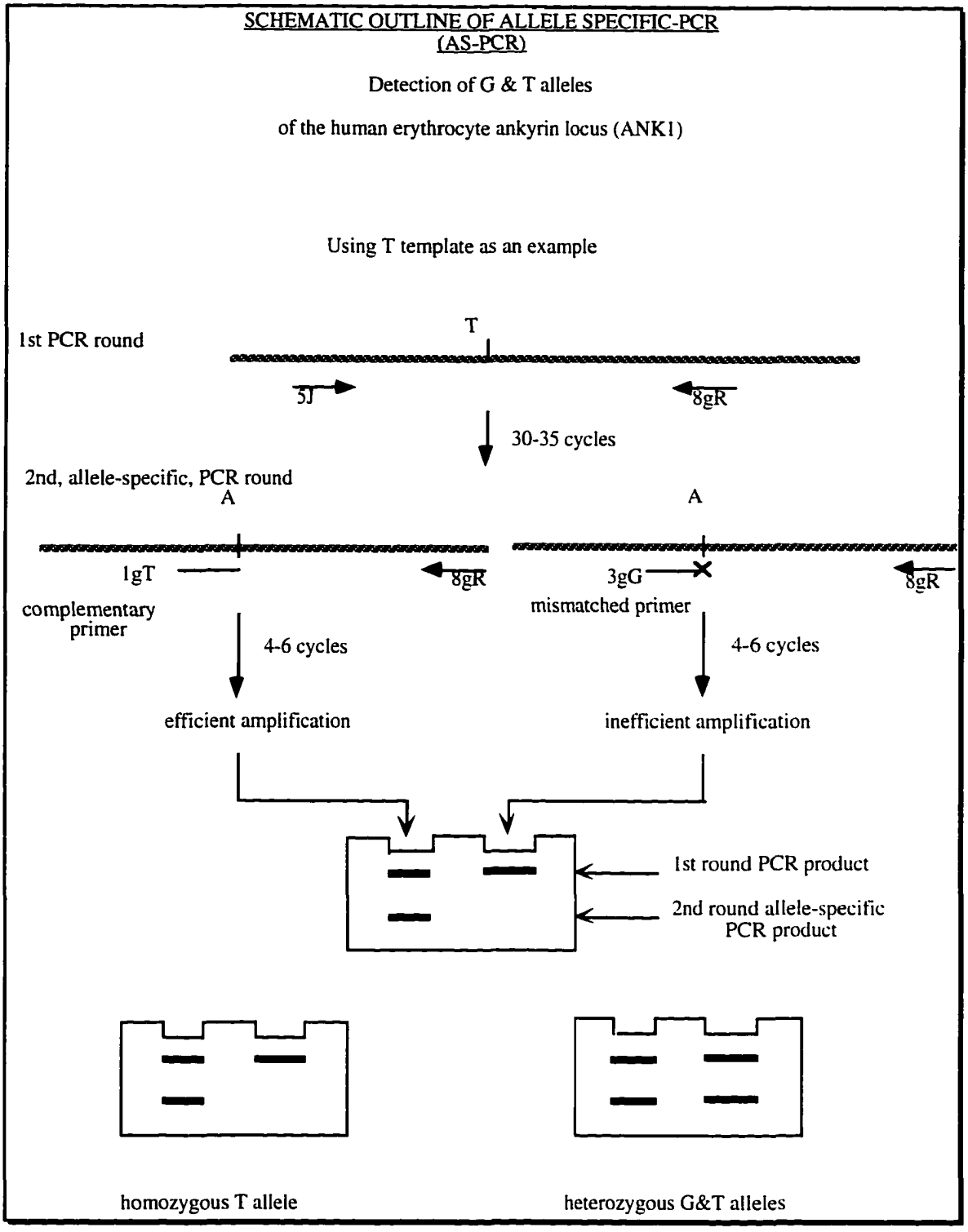

Figure 15 Schematic illustration of the allele-specific PCR (ASPCR) protocol. This procedure is utilized for the detection of identified nucleotide substitutions. (Courtesy of Dr. Yunying Liu)

(5) Allele-specific oligonucleotide: The design of the allele-specific oligonucleotide proved very critical. A T_m as low as 42°-44°C along with the mismatch being placed at the 3' base increased specificity. Whenever a segment could not efficiently be amplified, designing of a new set of primers using the other strand for allele-specific mismatch was suggested in order to provide better amplification.

(6) Deoxynucleotide concentration: Spurious amplification was prevented by decreasing the dNTP concentration to 25-50 mM.

(7) *Taq* polymerase: The amount of enzyme utilized in each reaction was typically decreased to 0.4-0.6 U per 50 µl reaction. This was recommended in order to increase specificity and decrease the direct cost of the assay. Higher concentrations of polymerase generated additional nonspecific bands. Such nonspecific bands could be used as internal controls in this assay.

(8) Formamide: Including formamide in the reaction, typically 2%-5%, was recommended as a means of increasing the strength of the signal and eliminating undesired bands particularly at high G+C content. Additionally, allele specificity could be enhanced.

(9) Additional pair of primers: Inclusion of a second pair of compatible, but nonspecific, primers could lead to the generation of a constant band, not at the allele-specific site. This could primarily serve the purpose of an internal control for the technical success of the PCR amplification. Furthermore, by providing a competing substrate for the *Taq* polymerase, the presence of this constant band could mean increased specificity.

(10) Source of DNA template: Using a PCR product as the source of DNA template for a nested ASPCR could increase specificity; this had also been suggested for highly repetitive sequences. In such a case, the DNA concentration was critical, and an additional 10^6-fold or even greater dilution would be required of the original PCR product.

(11) Number of amplification cycles: Decreasing the number of the cycles in the second round of PCR amplification could reduce the detection of any minor amplification of the mismatched allele. Increase, on the other hand, in the number of cycles could

generate fragments that would otherwise not amplify to detectable levels, and therefore complicate interpretation of the results.

(12) Annealing temperature: Raising the PCR annealing temperature could conceptually increase specificity by eliminating spurious amplification fragments. We never needed to vary the standard cycling times except in the case of the amplification of the genomic fragment of ankyrin where it was necessary to increase the elongation time and facilitate amplification of the relatively large fragment of about 1 kb.

Optimization of any of the above parameters could achieve the desired results of adequate amplification and detection of specific alleles. In our experience, all the combinations of mismatched primer and template pairs were successful.

Technically, ASPCR involved a simple two-step PCR reaction. The first round PCR generated high quality and concentration of the template that contained the substitution of interest. Total reticulocyte RNA was reversely transcribed to generate first strand cDNA using random hexamer primers, 5X reverse transcriptase buffer, DTT, and 1 μl of reverse transcriptase (GIBCO, BRL), in a total volume of 20 μl. Five μl of this cDNA reaction was then amplified by PCR using 25 pmol of any informative set of primers described in Table 3, 10X PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, and 1 unit of Taq polymerase. 35 cycles of 94°C for 40 sec, 58°C for 60 sec, 72°C for 2 min were performed in the GeneAmp PCR System 9600 (Perkin Elmer, CA). The second-round PCR involved amplification of the template of interest utilizing a total of three primers in two separate tubes. In both tubes the same reverse primer was used. The following forward allele-specific primers were used: (i) a forward primer specific for the mutation, and (ii) a forward primer specific for the wild type. Five μl of the first-round PCR product were amplified with 10 pmol of each of the primers: 4-6 cycles of 94°C for 30 sec, 60°C for 60 sec, 72°C for 1 min were performed in the same GeneAmp PCR 9600 System. Fifteen μl of the second round PCR product was analyzed on an 1% Agarose gel with 0.5 mg/ml ethidium bromide. In each of the reactions the products from the first round PCR were

visible, while the allele-specific products accumulated to detectable levels only in the tube that contained the perfectly matched primer-template set.

Assay of the Mutant Transcript

Was the mutant transcript always present in amounts that could consistently be assayed? This question became of great significance in this project in order to interpret some of the results obtained. This pertinent issue was determined to be addressed by means of obtaining an indication of the relative amounts of ankyrin transcripts derived from the two chromosomes. Advantage was taken of a previously described exonic dinucleotide $(AC)_n$ repeat polymorphism at the 3'-untranslated region (UTR) of the human ankyrin gene (Ank1) (Polymeropulos et al. 1991). This highly polymorphic dinucleotide repeat begins at base pair 6,304 of cDNA sequence of the human ankyrin gene on chromosome 8p11.1-21.1 (Lux et al. 1990a). Additionally, it has been reported that a comparison of this $(AC)_n$ microsatellite repeat in both genomic DNA and mRNA can be used to study the expression of erythroid ankyrin (Jarolim et al. 1995b).

The haplotype of this repeat can be ascertained using PCR, with a predicted length of 109 bp. Estimated from 50 chromosomes of unrelated individuals, the observed heterozygosity was 54%. Table 9 summarizes the four different alleles along with their observed frequencies and sizes. A co-dominant segregation in two informative families was reported by the same group (Polymeropulos et al. 1991).

Amplification of the $(AC)_n$ Repeat Polymorphism

Genomic DNA was isolated from the propositus and PCR was performed using 10 pmol of primers: *3'UTRfor* (5'-TCCCAGATCGCTCTACATGA-3', nt 6,276-6,295) and *3'UTRrev* (5'-CACAGCTTCAGAAGTCACAG-3', nt 6,385-6,366), (Lux et al. 1990a). The standard components included the 10X buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, and 1-2 U of *Taq* polymerase in the presence of [^{32}P]-(dATP). Thirty-five cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 1 min were performed. An initial denaturation and a final extension step were included in the program. The PCR was performed in the GeneAmp

PCR System 9600 (Perkin Elmer, CA). The PCR products were electrophoresed in the Base Runner Nucleic Acid Sequencer (International Biotechnologies, New Haven, CT) through a 6% denaturing gel at 45 W for 3 h at room temperature and exposed to X-OMAT Imaging Film (Kodak, Rochester, NY) at -80°C for 15 min. Alternatively, PCR was performed under the same cycling conditions in the absence of radiolabeled dATP, and the products were electrophoresed in the SE 600 vertical slab gel unit (Hoefer Scientific, San Francisco, CA) for 7 h at 150 V at room temperature through a 1.5 mm-thick 12% nondenaturing gel and stained with 10 mg/ml ethidium bromide for 30 min.

Comparison of the $(AC)_n$ Microsatellite Length in Genomic DNA and cDNA

Total reticulocyte RNA was isolated from the propositus, reverse transcribed to cDNA using random hexamer primers and PCR amplified using the same conditions as for genomic DNA. The PCR products from genomic DNA as well as cDNA were loaded side by side and electrophoresed, and the patterns obtained from genomic DNA and cDNA were compared.

Table 9

Dinucleotide Repeat Polymorphism Characterization.

Allele	# bp	# Repeats	Frequency
A1	113	14	0.40
A2	111	13	0.04
A3	109	12	0.02
A4	107	11	0.54

RESULTS

Family Pedigree Study: Clinical Data

For all the clinical data concerning the proband see Case Report on page 23. The pedigree of this family is outlined in Figure 16. Individuals II:5, and II:7, the sister and brother of the propositus, and III:4 and III:5, the offspring of II:7, were initially thought not to have spherocytosis on the basis of normal hemoglobin and reticulocyte count. Initial hematological evaluation of individual II:5 revealed a hematocrit of 41%, hemoglobin levels of 14.1 g/dl, and a reticulocyte count of 1.0%, all within the normal range. Likewise, the initial hematological examination of II:7 was normal, with a hematocrit of 46% and hemoglobin levels of 16.4 g/dl.

During the course of this project, individual III:2, the 28-year-old niece of the propositus, was found to be significantly anemic during her pregnancy, with a hematocrit of 24.4%. Further hematologic analysis revealed an extremely low serum haptoglobin level, a high reticulocyte count, and peripheral blood smear consistent with significant macrocytosis and spherocytosis. Osmotic fragility was significantly increased with initial hemolysis of incubated blood at 70% NaCl and complete hemolysis at 60% NaCl, compared to 55% and 30% NaCl in control material, respectively.

These recent clinical findings mandated the medical re-evaluation of II:5 and II:7, upon which it was revealed that they had nonanemic mild spherocytosis as documented by the presence of spherocytes on their blood film and abnormal osmotic fragility tests. More specifically, osmotic fragility tests performed on 24-hour incubated blood of individual II:5 revealed initial hemolysis at 60% NaCl and complete hemolysis at 45% NaCl, as compared to 55% and 30% in the control, respectively.

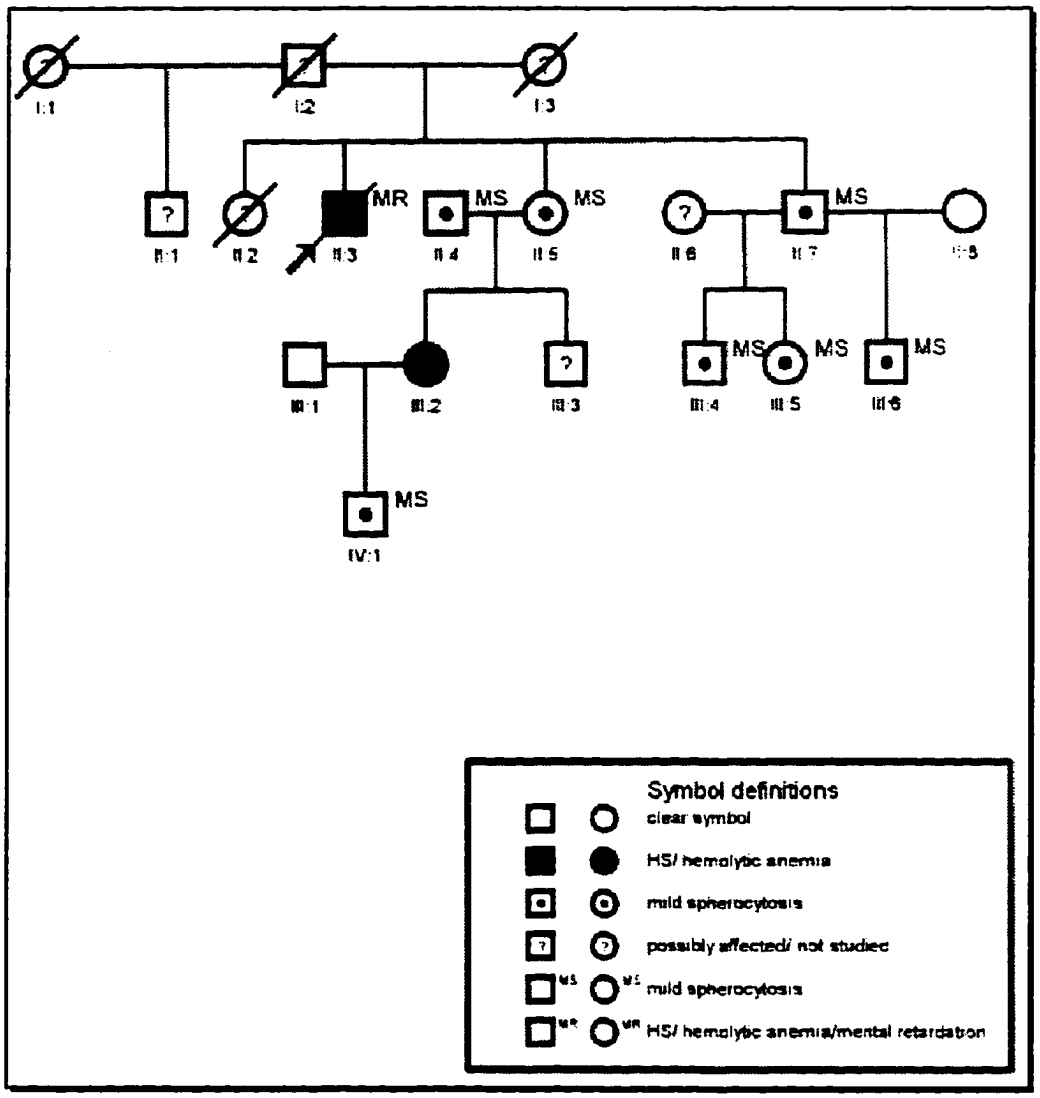

Figure 16 Family pedigree: Hereditary Spherocytosis.

Individual II:4, the father of III:2, was also found to have nonanemic mild spherocytosis; consanguinity was reported to be excluded, although paternity tests had not been performed. In addition, individuals III:4, III:5, III:6, as well as the son of III:2, individual IV:1, had spherocytes on their blood film. III:4 had a slightly elevated hematocrit been performed. III:4 had a slightly elevated hematocrit of 50%; on the other hand, hematologic evaluation of III:5 revealed a low hematocrit (37%), low hemoglobin levels of 12.1g/dl and low red blood cell (3.77 MI / CMM) and white blood cell (3,310 cells / CMM) counts. Both III:4 and III:5 have been found to have abnormal osmotic fragility. Hematologic analysis of individual III:6 showed a slightly lower hematocrit of 38% and a hemoglobin level of 14.4 g/dl, at the lower side of the normal range.

Total peripheral blood had been collected in heparin-containing tubes from individuals II:3, II:4, II:5, II:7, II:8, III:2, III:5, III:6, and IV:1; subsequently, genomic DNA and reticulocyte RNA had been isolated from all these individuals.

Northern Blot Analysis

Total reticulocyte RNA was isolated from the propositus and an unrelated normal control using the method of guanidine isothiocyanate originally described by Chromczynski (Chromczynski and Sacchi 1987) and subsequently analyzed by Northern blot hybridization. This demonstrated the presence of two distinct species of mRNA of approximately 7.2 and 9.0 kb in size in the propositus (II:3) as well as the control, as previously reported (Lambert et al. 1990; Hanspal et al. 1991) (fig. 17). The radioactively labeled probe that was used in the hybridization reaction was p(10-3') (fig. 9). The amounts of the transcripts were objectively evaluated in a phosphorimager (Biochemistry and Molecular Genetics, UAB). Upon quantitation of the intensity of the signals seen in the propositus in comparison with the normal controls, a 50% decrease in the signal intensity was confirmed. The high molecular weight transcript was less abundant in the proband. The amounts of RNA analyzed, were calculated by spectrophotometric analysis and by visualization on 1% Agarose gels.

Alternative mRNA Processing of Erythrocyte Ankyrin

The ratio of the 7.2-kb/9.0-kb erythroid ankyrin transcripts was significantly increased in the propositus to a value of 10.83 ($p<0.009$) compared to 6.15 in adult human reticulocytes, while the larger size transcript was notably decreased.

5'-RACE

By means of 5'-RACE, the possibility of alternative mRNA processing at the 5'-UTR of the ankyrin gene was examined as a potential mechanism underlying the increase in the two ankyrin transcripts ratio. Total reticulocyte RNA was isolated from the propositus and a normal control, reverse transcribed using the ankyrin cDNA-specific reverse transcription oligonucleotide primer (RT-1): 5'-CCTTCCTTAGAAGCCAGATGCAAGC-3'. The anchor primer was then ligated to the cDNA and PCR was performed using a nested ankyrin-specific primer (TA-2): 5'-GTTACCTGATCTTGCTGCTCTC-3' and another primer whose sequence was derived from the sequence of the anchor that was attached to the 3'-end of the first-strand cDNA: 5'-CCTCTGAAGGTTCCAGAATCGAT AG-3'. This analysis did not reveal any differences between the proband and normal controls in the PCR amplification patterns obtained. Subcloning and sequencing of the 5'-RACE products did not reveal the presence of a mutational event in that region of the human erythrocyte ankyrin gene in the proband. The sequencing results confirmed the published sequence of the ankyrin mRNA; the possibility of any alternate pattern of mRNA processing occurring in the propositus was ruled out (fig. 10, 11, and 18) and (table 4).

3'-RACE

The technique of 3'-RACE was used to analyze the 3'-UTR of the ankyrin gene in the propositus and normal controls. Total reticulocyte RNA was isolated from the proband and controls, and was reverse transcribed to cDNA using a 57-bp oligonucleotide primer, containing 17-T residues along with a randomly assigned sequence that was used to facilitate design of two primers to be used in the subsequent PCR-amplification reactions.

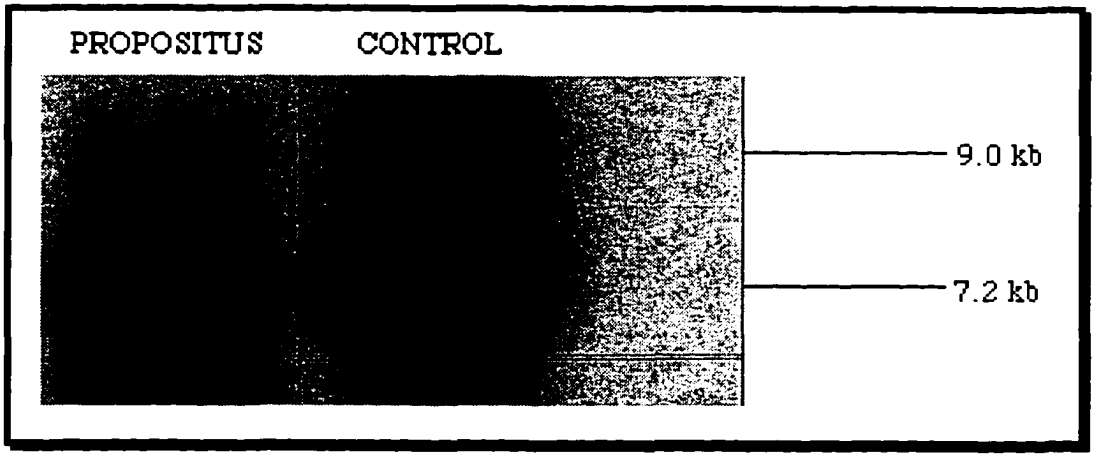

Figure 17 Northern Blot Analysis. Total reticulocyte RNA from the propositus (CC) and a normal unrelated control was analyzed with a human erythrocyte ankyrin (ANK1) cDNA probe. The probe, p (10-3'), is a 1.5-kb cDNA fragment, close to the 3'-end of the ankyrin cDNA. The autoradiogram was exposed for 10 days in order to obtain a clear picture of the higher molecular weight ankyrin transcript.

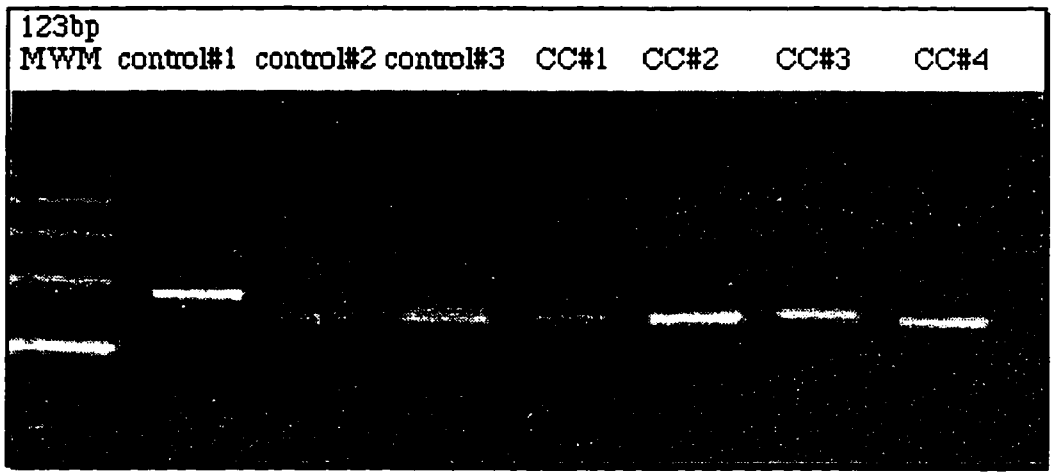

Figure 18 Results of 5'-RACE end analysis. Four separate samples from the propositus, and three unrelated controls were analyzed. 5'-RACE analysis was followed by subcloning and sequencing of the RACE products. The data revealed neither any differences in the amplification patterns nor in the primary nucleotide sequence of the propositus, ruling out the possibility of alternative mRNA processing in the 5'-end of the ANK1 gene and the presence of mutations in the same region.

The first strand cDNA was then PCR-amplified in two rounds of amplification using the two pairs of outer and then inner (nested) primers (table 5). The 3'-RACE analysis did not reveal any differences among the propositus and unrelated normal controls; the PCR-amplification patterns that we observed in the propositus were consistently similar to the ones from the controls and there was no evidence indicative of alternate processing of ankyrin mRNA (fig. 19) and (table 5).

Alternative modes of exon splicing?

Based on the published sequence of human erythrocyte ankyrin (ANK1), nine pairs of overlapping primers that specifically PCR amplified ~7.25 kb of the ankyrin ORF (5.6 kb) and UTRs (~85 bp in the 5'-UTR and ~1,525 bp in the 3'-UTR) were designed (table 6) (Lambert et al. 1990; Lux et al. 1990a) (fig. 14). Fragments of cDNA, derived from PCR amplification of reversely transcribed total reticulocyte RNA using these nine sets of primers, were of the expected size and no differences were observed in the amplification patterns in the propositus and various unrelated normal controls. Thereby the possibility of a major gene abnormality, such as deletion of a large part of the coding sequence of the gene, any other rearrangement occurring in the propositus, or both was eliminated. Moreover, the data ruled out any prospect of alternative mRNA processing occurring in the open reading frame (ORF) of the ankyrin gene that might explain the significantly increased ratio of the two erythroid ankyrin transcripts (7.2 kb/9.0 kb) to a value of 10.83 ($p<0.009$) compared to the normal control value of 6.15 in adult human reticulocytes.

Interestingly, when PCR was performed using the *6FOR* (5' at 4,127) and *7REV* (3' at 5,968) set of primers, that should amplify a fragment of 1,861 bp in expected size, two bands were actually generated in both the propositus and normal controls, even when the reaction was performed at a high annealing temperature (65°C), using several different normal controls (fig. 20). The second band was approximately 1.37-kb in size. This evidence was consistent with previous reports of alternative mRNA processing that appeared to be occurring at that region of the ankyrin gene, with 486 bp missing from some

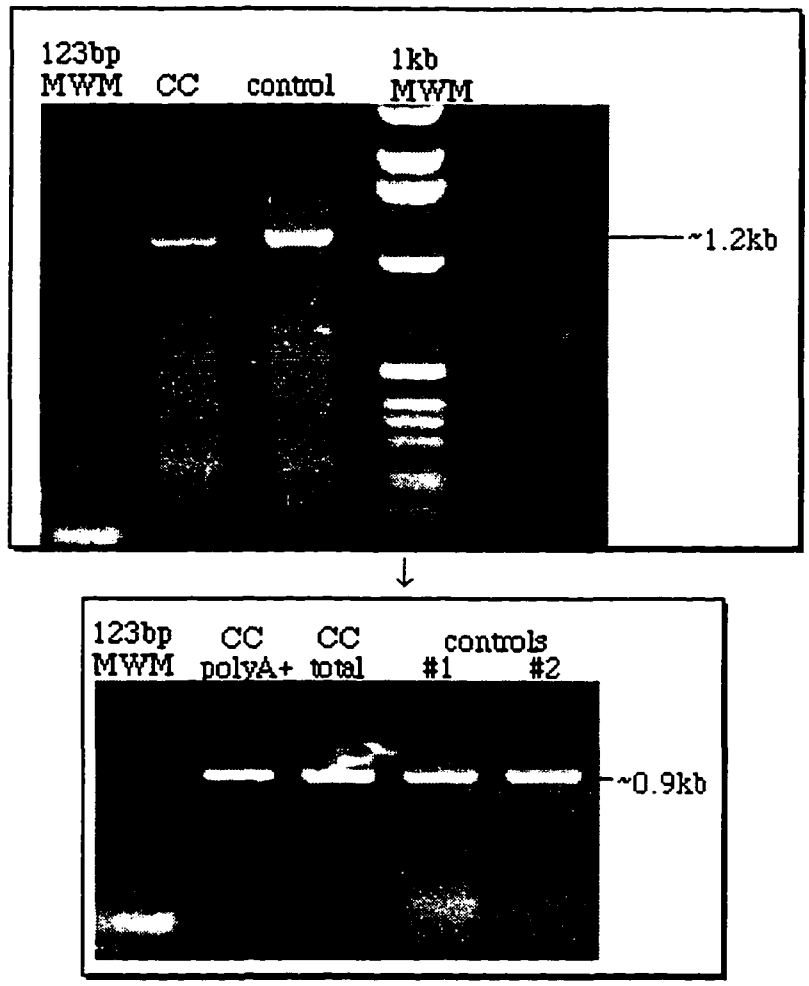

Figure 19 Results of 3'-RACE end analysis. The upper panel illustrates the amplification pattern of the first-round PCR, in the propositus (CC) and a normal unrelated control, while the lower panel shows the results of the second-round PCR. The pattern obtained when using total reticulocyte RNA as the starting material did not differ from the ones with poly(A)$^+$ mRNA. The difference in the size of the PCR product is explained by the fact the two GSPs are separated by 280 bp of sequence. No differences in the patterns obtained in the propositus and normal unrelated controls rule out any alternate modes of processing at the 3'-end of the ANK1 gene.

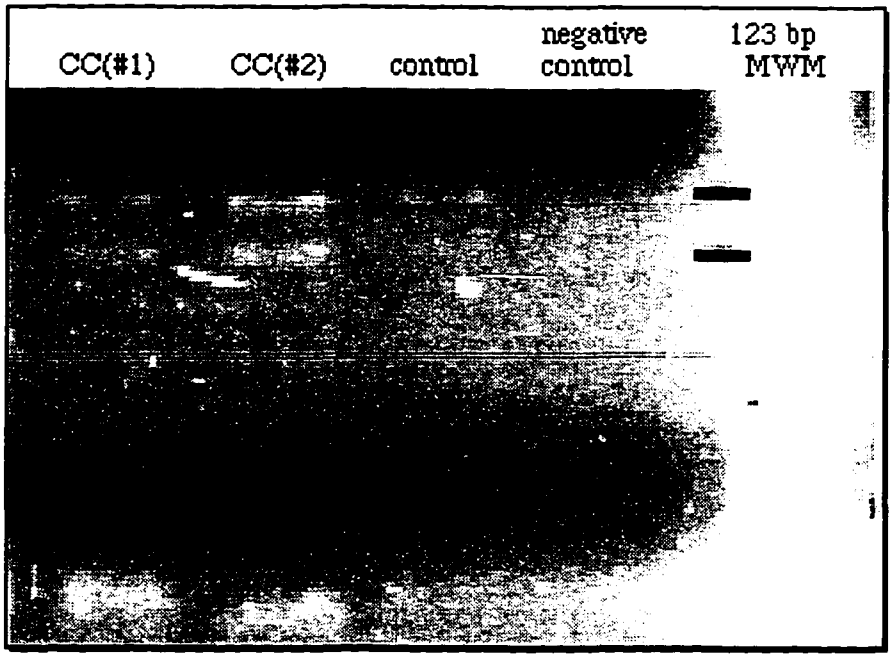

Figure 20 Amplification results using the primer set *6for/7rev*, indicating alternative mRNA processing of ANK1. This has been known to occur at that region of the ANK1 gene, explaining the "2.1" from the "2.2" ankyrin isoform.

overlapping ankyrin cDNA clones, leading to the in-frame deletion of 162 amino acids. These 486 bp constitute a major portion of exon 38 and their absence has been known to generate a different isoform of ankyrin (ankyrin 2.2) with major functional differences compared to the ankyrin 2.1 isoform; ankyrin 2.2 is an "activated" form with a three-fold higher affinity for spectrin, as well as the ability to bind twice as many band 3 molecules (Lambert et al. 1990; Lux et al. 1990a; Peters and Lux 1993).

SSCP Analysis

SSCP Strategy

Since the presence of a major deletion or rearrangement of the ankyrin coding region was not revealed, the possibility of a small deletion or single nucleotide substitution was explored by SSCP analysis. The entire coding sequence of ankyrin was screened using SSCP. Nine fragments ranging in size from 0.8-1.2 kb were PCR-generated using nine overlapping pairs of ankyrin cDNA-specific oligonucleotide primers (table 6). All fragments were gel-purified using the QIAEX II Gel extraction kit (QIAGEN kit) for further analysis. Nine combinations of multiple restriction endonucleases were chosen to yield the optimal size fragments for SSCP analysis (150-250 bp).

SSCP Results

The presence of two putative DNA changes was manifested as consistently reproducible mobility shifts in two different cDNA fragments (fig. 21 and 22). This suggested that the abnormal SSCP pattern observed in the propositus might result in the presence of mutation(s). One was created using the *2FOR/2REV* set of primers, 788-1,711 bp, and *Msp*I restriction endonuclease, (fig. 21), while the other was created using the *4FOR/4REV* set of primers, 2,534-3,262 bp, and *Dde*I restriction enzyme digestion (fig. 22) and (table 6). Our study outline was designed to lead to the confirmation of these suspected DNA changes, their delineation by sequencing, and their ultimate discrimination whether they were non-specific polymorphisms also occurring in the general population or disease-specific, and possibly even, this HS family-specific.

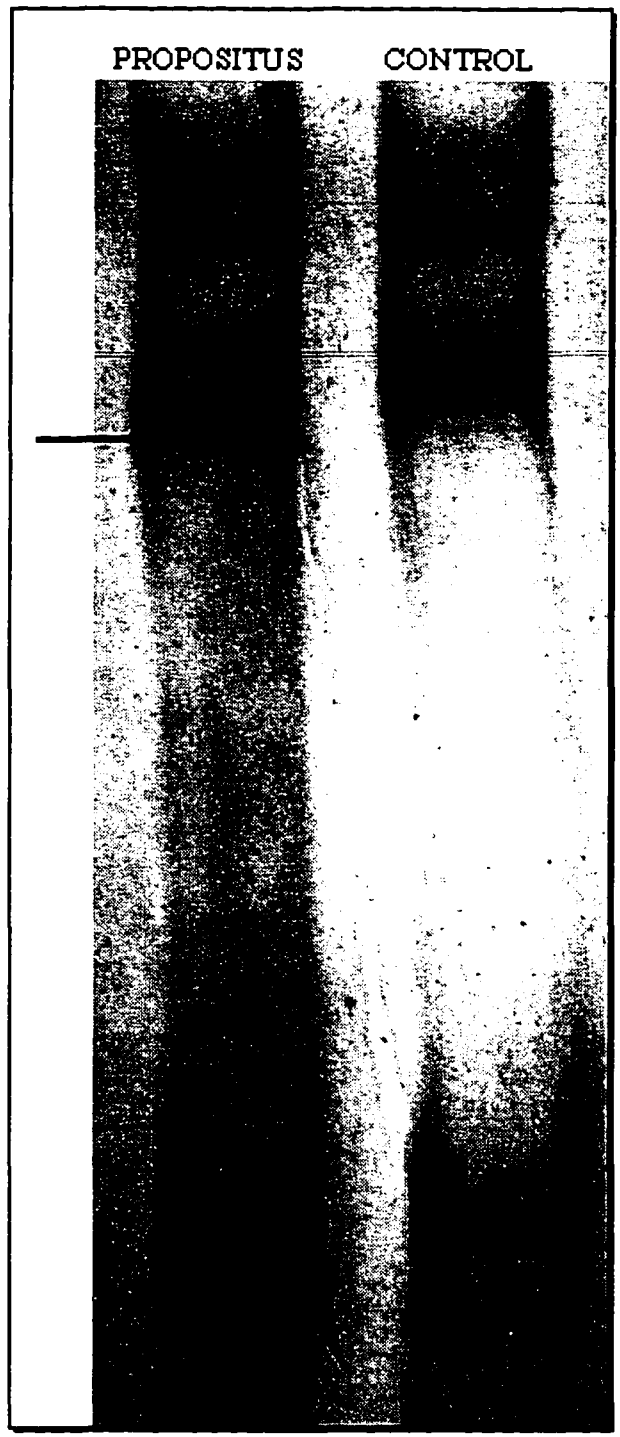

Figure 21 SSCP analysis of the *2for/2rev* fragment digested with *Msp*I restriction endonuclease. Three individual samples from the propositus are compared side-by-side with three unrelated normal controls, yielding a consistently reproducible, band mobility shift, indicative of underlying DNA changes.

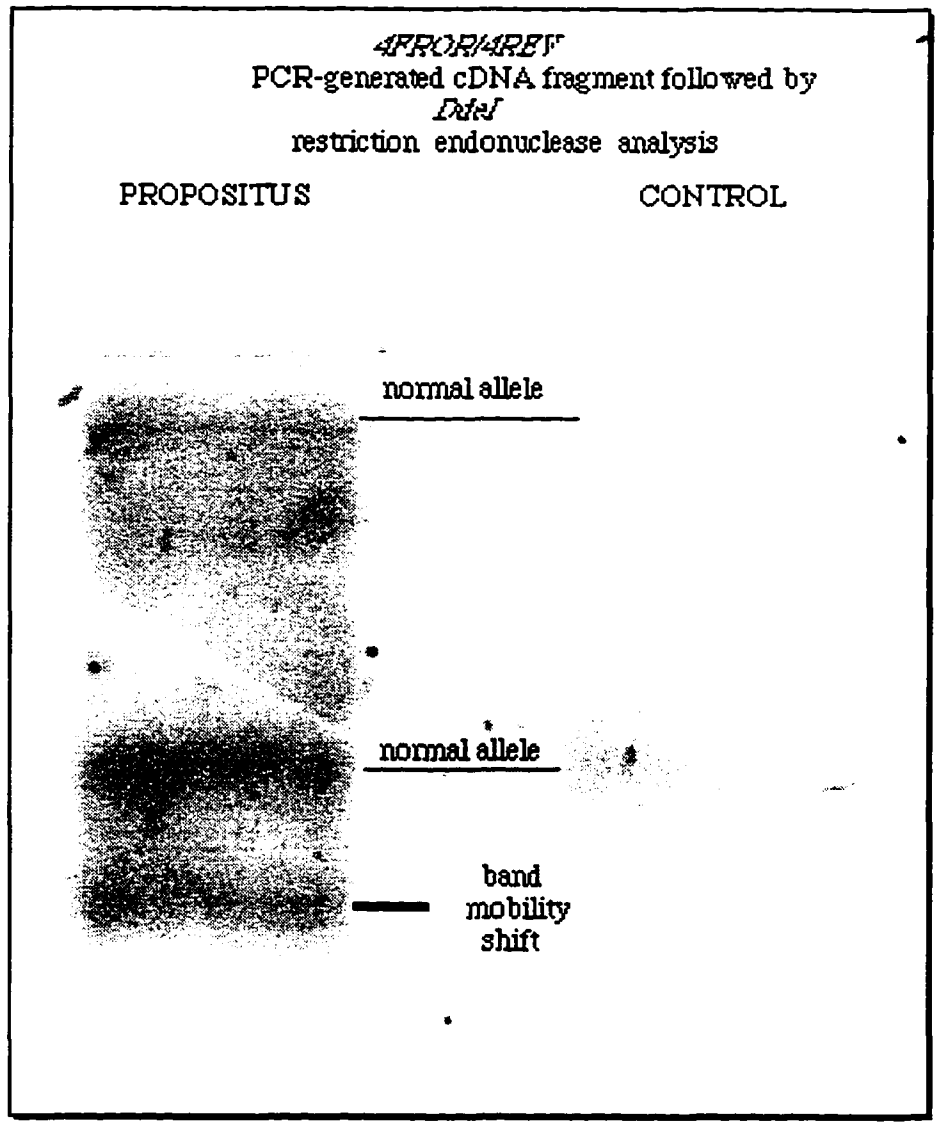

Figure 22 SSCP analysis of *4for/4rev* digested with *Dde*I restriction endonuclease. The propositus is compared side-by-side with an unrelated normal control, yielding a band mobility shift that was again indicative of potential underlying DNA changes.

The two fragments of interest were subcloned into pCRII, and clones with the correct-size insert were analyzed by SSCP using the same restriction enzymes that had resulted in the original mobility band shifts (fig. 23). Two clones of the *2for/2rev* fragment revealed similar shifts, when compared to the digests that had revealed the change in the migration pattern in the propositus, and their respective plasmids were subsequently sequenced. . Analysis of the *4for/4rev* clones did not reveal any changes in the migration pattern when compared to the original digests; this fragment was sequenced nevertheless. One of the normally migrating clones was sequenced to serve as a normal standard.

Nucleotide Sequence Analysis: Results

Both the sense and anti-sense strands of the two abnormally migrating clones of *2for/2rev*, designated as *1CC* and *5CC*, were sequenced (table 8). No changes were revealed in the primary nucleotide sequence of *5CC*. On the other hand, nucleotide sequence analysis of *1CC* revealed a C-to-T transition at position 1,270, resulting in a serine-to-proline change at position 396 (S396P). Additionally, an A-to-G transition at position 1,324, changed a methionine to a valine (M414V) (fig. 24).

Confirmation of Nucleotide Changes

The next question to be answered was whether these substitutions were real or potential artifacts of the PCR reaction (*Taq* polymerase lacks the 3'Æ5' exonuclease proofreading function and has an error rate, error/bp incorporated, of 7.2×10^{-5} at best).

Sequencing of PCR Products

Sequencing of PCR products was pursued as an alternative because it offered the advantage that artificial nucleotide changes were statistically less likely to occur. Products of the same region of cDNA but of different PCR amplification reactions were sequenced. The changes that were previously revealed were not present in the fragments that were analyzed. Sequencing was performed in both orientations; in addition, in order to eliminate compression problems encountered, the analog-7-deaza-dGTP was utilized in the sequencing reactions.

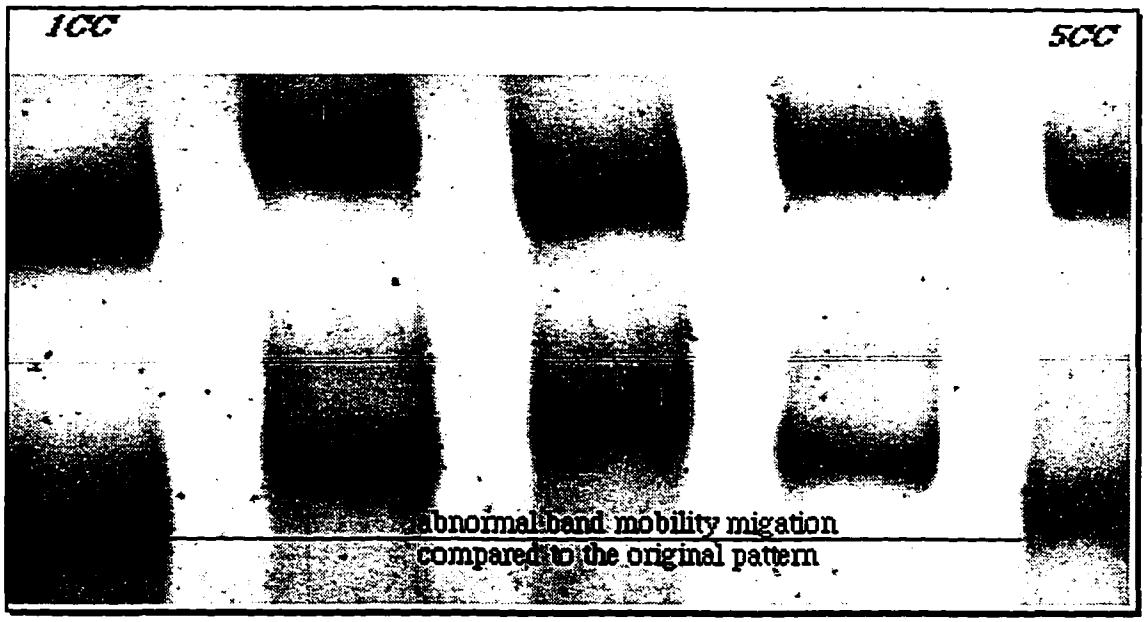

Figure 23 SSCP analysis results of the candidate clones of *2for/2rev* digested with *Msp*I. Identification of suspected nucleotide substitutions manifested as band mobility shifts was followed by subcloning of the fragments of interest and further analysis. First, the candidate clones were analyzed by SSCP using the same restriction endonucleases that revealed the original mobility shifts. Clones that migrated with similar patterns when compared to the original digests that had originally revealed the changes in the migration pattern, were chosen to be further analyzed. The figure shows SSCP analysis of *2for/2rev* clones further digested with *Msp*I, the original endonuclease of interest. The lanes marked as *1CC* and *5CC* showed band mobility shifts in their inserts and were further sequenced.

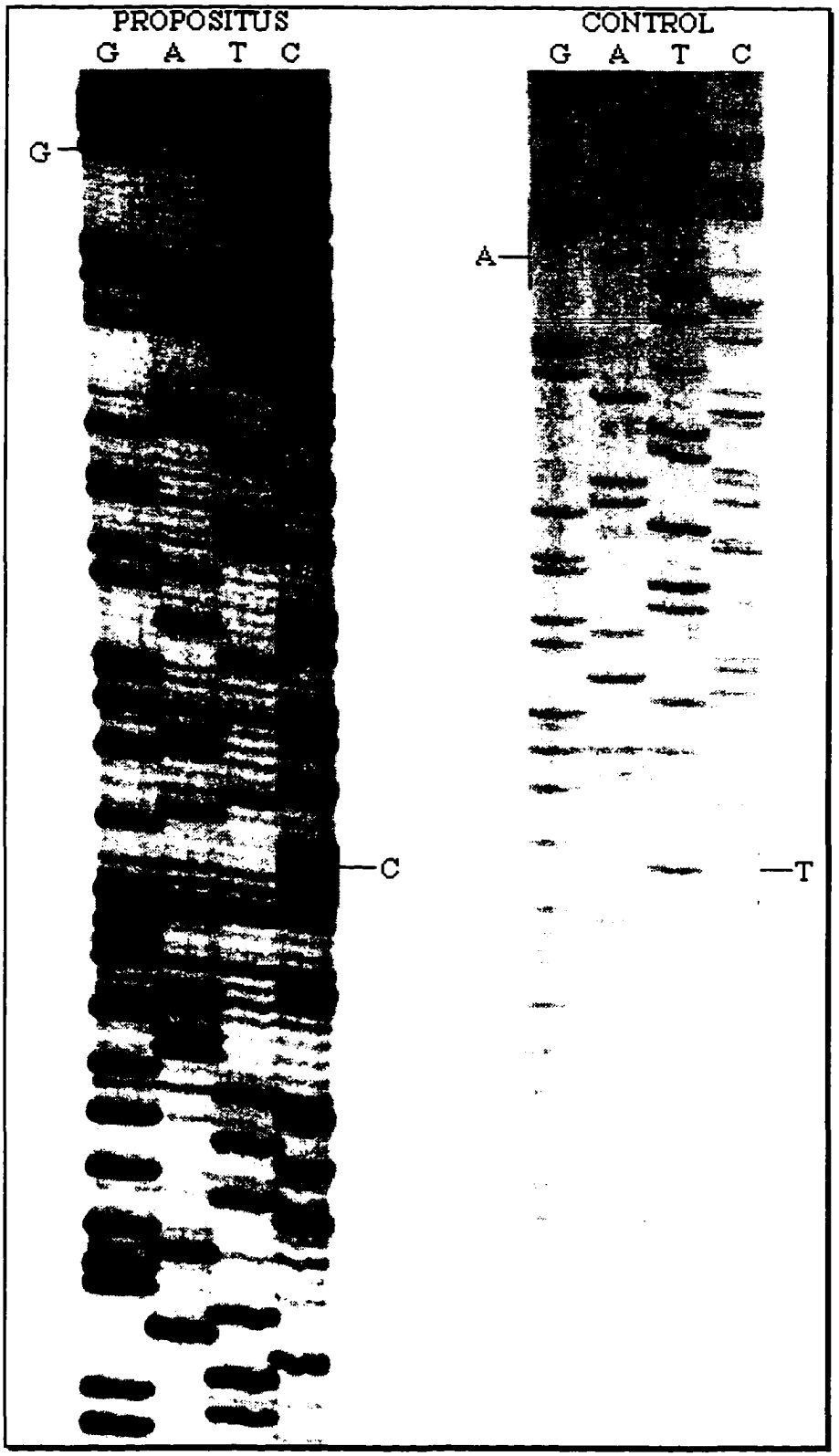

Figure 24 Sequencing results. Sequencing of the *2for/2rev* cDNA fragment of clone *1CC* was performed using primer $2for_B$. The results revealed a C-to-T transition at position 1,270, and an A-to-G transition at position 1,324.

At that point, interpretation of these findings was sought for, and the following two issues were addressed: (i) were these findings an artifact of PCR and/or cloning and sequencing, and (ii) compared to the normal transcript, was the mutant transcript present in sufficient amounts that could be consistently assayed for.

Allele-specific PCR (ASPCR)

It was then determined whether ASPCR was an effective means to confirm the presence of the mutations under study. When the normally migrating clone was analyzed by ASPCR , the sequence was normal. Similarly, when the abnormally migrating *1CC* was analyzed by ASPCR, the suspected mutations could be assayed for. In contrast, newly generated PCR products of the same ankyrin cDNA region failed to reveal the changes under analysis. We have therefore concluded that the suspected nucleotide changes were not present in cDNA from the propositus (fig. 25 and 26).

The presence of the nucleotide substitutions under question was next examined in genomic DNA. This would unequivocally establish their status. Dr. Bernard Forget, from Yale University, provided us with the previously unpublished intron/exon boundaries of the ankyrin gene at the particular region of interest. The specific exon under study (exon #12, 1,290-1,390) was amplified using genomic DNA-specific oligonucleotide primers, *gen2for/gen2rev* and yielded an ~1.2 kb fragment (fig. 27). ASPCR analysis of this genomic DNA-generated fragment that contained both sites under study did not reveal the presence of the suspected changes. The genomic fragment of the normal control was also normal by ASPCR; the substitutions in question were absent (fig. 28).

Additionally, extensive sequencing in both orientations of subcloned, as well as nucleotide sequencing of PCR products, with and without 7-deaza-dGTP incorporation, of the *4for/4rev* fragment did not reveal any nucleotide substitutions in its primary sequence.

The above data could be explained by the fact that the mutant transcript was not represented in adequate amounts to be assayed for consistently. To examine this possibility and determine whether both ankyrin loci were expressed, the relative proportion of the

ankyrin transcripts generated from the two chromosomes was estimated. Advantage was taken of the presence of a previously described exonic $(AC)_n$ repeat polymorphism in the 3'-UTR of ankyrin mRNA (Polymeropoulos et al., 1991) and the report that a comparison of the ankyrin $(AC)_n$ microsatellites in both genomic DNA and mRNA could be used to study erythroid ankyrin expression.

Analysis of the $(AC)_n$ Repeat Polymorphism and Comparison of the Repeat Length in the Genomic DNA and cDNA

Total reticulocyte RNA was isolated from the propositus, reverse transcribed using random hexamers and PCR-amplified using the same conditions as for genomic DNA. The PCR products from both genomic DNA and cDNA were then electrophoresed side-by-side in 12% non-denaturing gels to compare the patterns of genomic DNA and cDNA. Heterozygosity for the ankyrin AC repeat-length was readily demonstrable, first in genomic DNA, with two loci being present in similar proportions; subsequent analysis of the AC repeat-length in cDNA demonstrated that the proband was heterozygous for this repeat polymorphism. This data was indicative of the fact that both ankyrin alleles appeared to be transcribed normally (fig. 29 and 30).

Although the resolution of the different sizes of the PCR products was much better in the autoradiogram (fig. 30), shadow bands were present along with the main PCR bands. This had been a commonly encountered phenomenon observed during haplotype determination of dinucleotide and trinucleotide repeats (Litt et al. 1993), most likely occurring due to slipped mispairing during the PCR amplification reaction (Litt et al. 1993). Because of the weak intensity of the shadow bands relative to the principal PCR bands, the presence of the shadow bands did not interfere with the interpretation of the results. The shadow bands were not present in the lower resolving capacity, smaller ethidium bromide-stained gels (fig. 29).

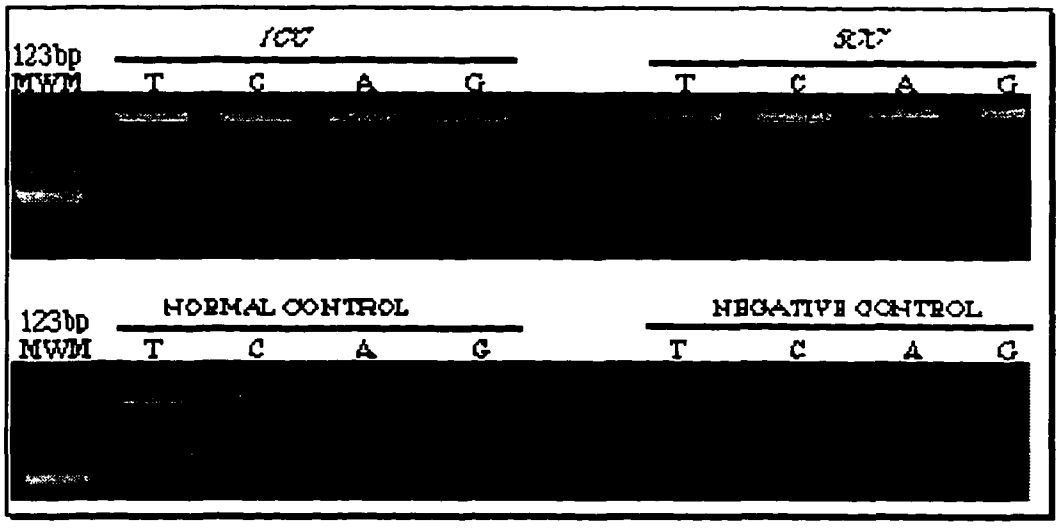

Figure 25 Results of AS-PCR analysis of the clones with the suspected changes. Lanes T and A contain the perfectly matched primers, while lanes C and G contain the mismatched primers. The results in all three clones confirmed the results obtained from sequencing.

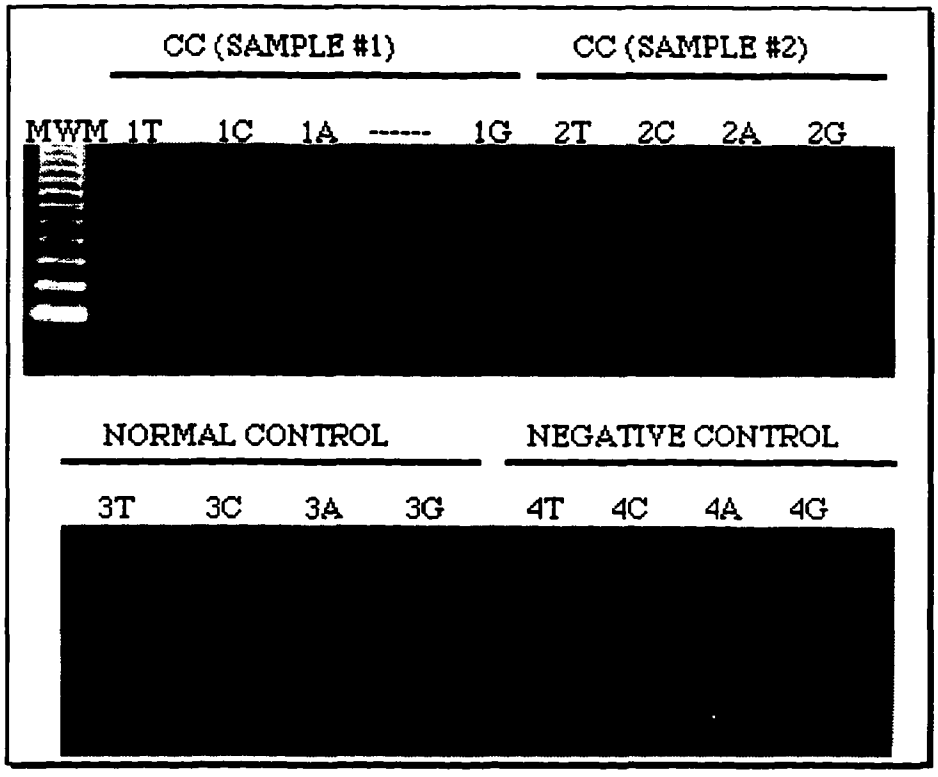

Figure 26 Results of ASPCR analysis of separate PCR generated *2for/2rev* fragments. Two different samples from the propositus (CC) were compared with the normal unrelated control. Lanes T and A contain the perfectly matched primers, while lanes C and G contain the mismatched primers. The data indicated that AS-PCR analysis of these fragments was normal, ruling out the presence of the suspected changes in the propositus.

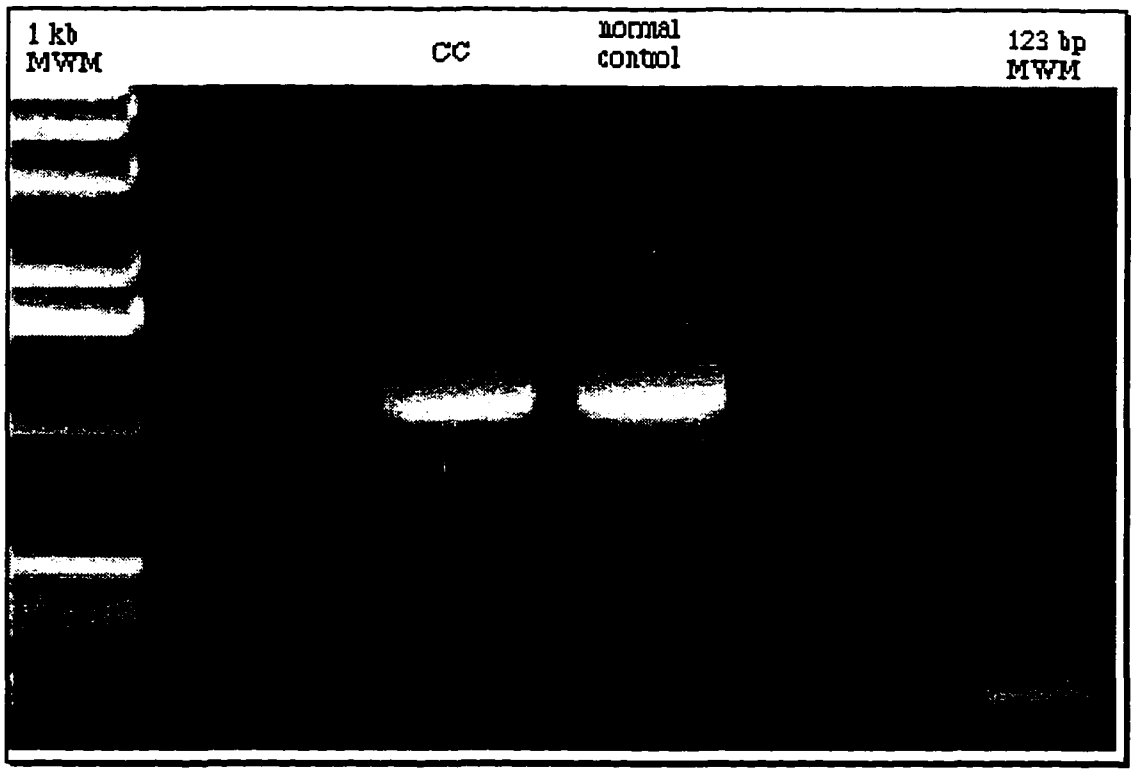

Figure 27 PCR amplification results of genomic DNA of the region of interest. The propositus was again compared to the unrelated control, and further analyzed by AS-PCR to investigate the presence of the suspected changes in genomic DNA from the propositus and unequivocally establish their presence or absence. The expected size of the PCR fragment was approximately 1.2 kb. The intron/exon boundaries of ANK1 are not published yet, so the exact size was unknown. Information was provided from Dr. Bernard Forget, of Yale University School of Medicine.

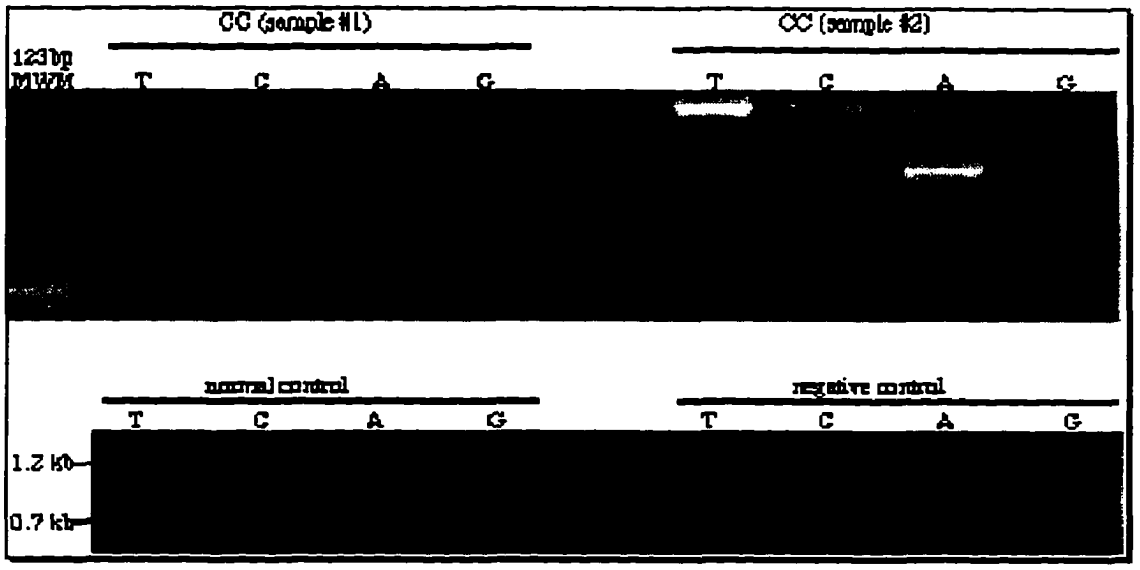

Figure 28 Results of ASPCR analysis of the genomic DNA-derived *2for/2rev* fragments. The presence of the suspected mutations was investigated in genomic DNA. Lanes T and A contain the perfectly matched primers, while lanes C and G contain the mismatched primers. The results showed absence of the suspected mutations in the propositus

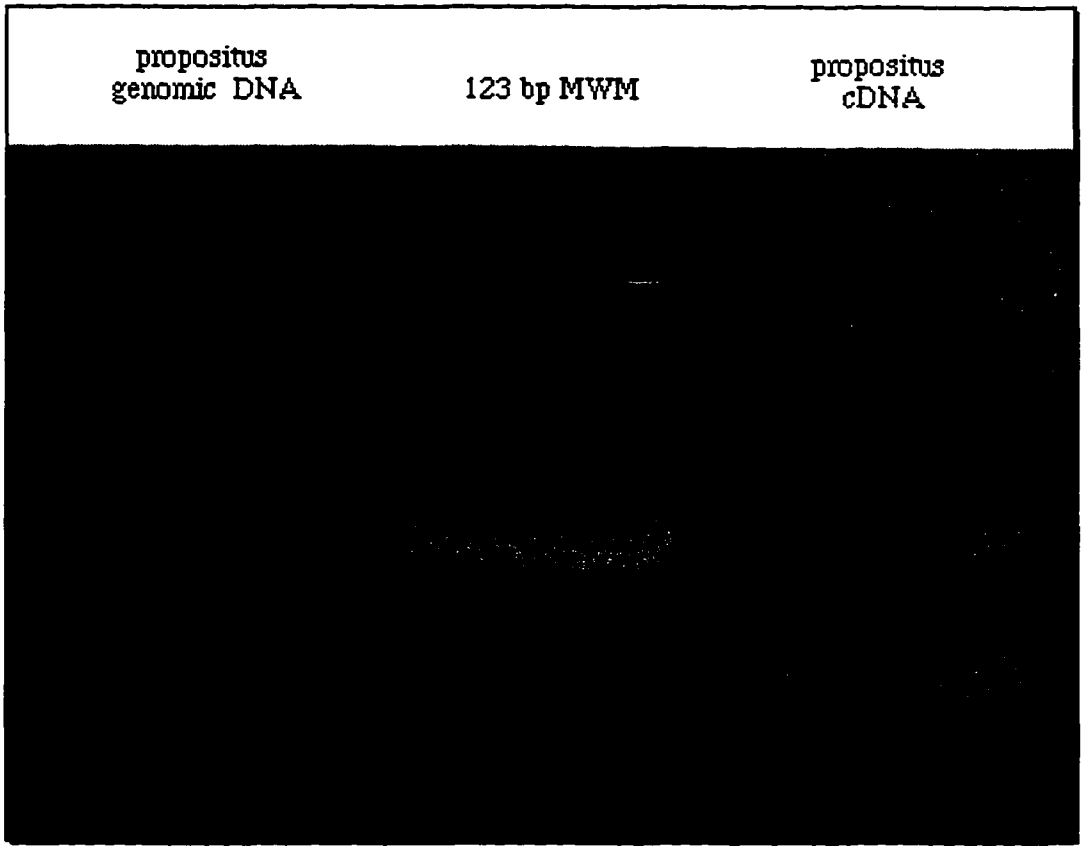

Figure 29 Analysis of the $(AC)_n$ repeat polymorphism and comparison in genomic DNA and cDNA in the propositus: Ethidium bromide stained gel. The shadow bands that were present in the autoradiograms are not evident here. Heterozygosity for the ankyrin AC repeat length polymorphism was readily demonstrable in both genomic DNA and cDNA in the propositus. This was indicative of the fact that both ankyrin alleles appeared to be transcribed normally.

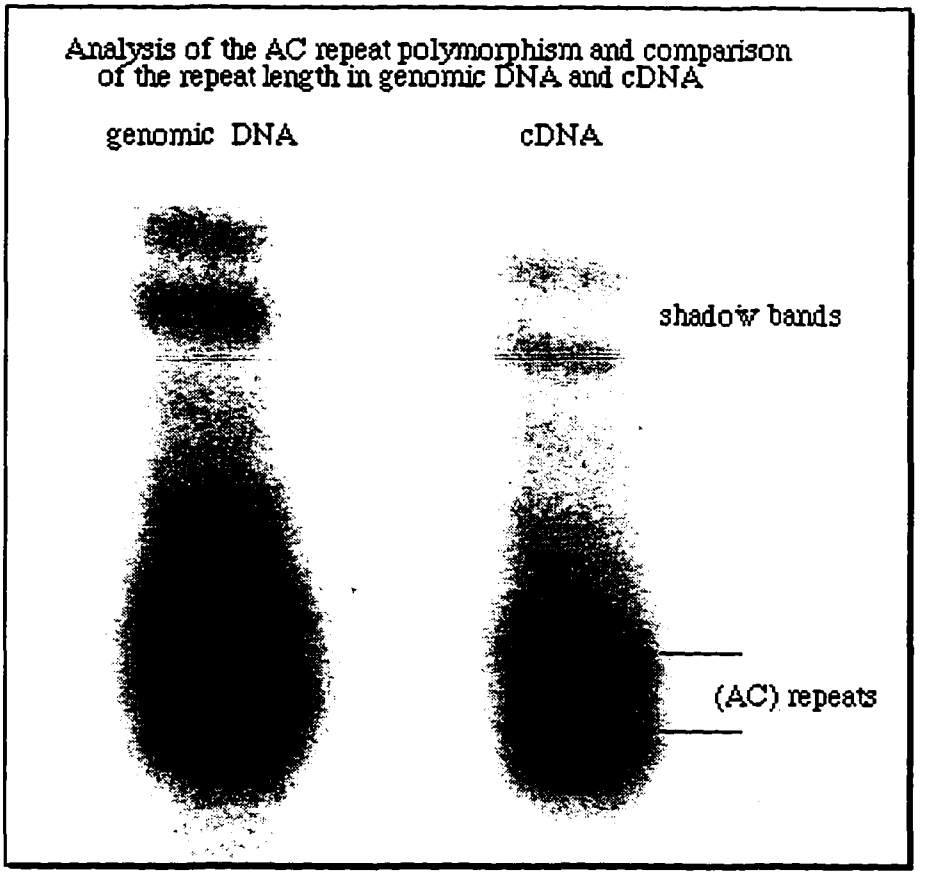

Figure 30 Analysis of the $(AC)_n$ repeat polymorphism and comparison in genomic DNA and cDNA in the propositus: Autoradiogram of PAGE. The PCR reaction was performed with the incorporation of radioactive material and the gel was exposed autoradiographically. In this case, shadow bands are evident because of the higher resolving capacity of this system. Heterozygosity for the ankyrin AC repeat length polymorphism was again readily demonstrable in both genomic DNA and cDNA in the propositus, indicative of the fact that both ankyrin alleles appeared to be transcribed normally.

DISCUSSION

In this family pedigree of varying degrees of inherited spherocytosis with or without hemolytic anemia, 11 potentially informative meioses for the study of HS have been identified. The propositus, individual II:3, was first diagnosed with life-threatening severe hemolytic anemia, which improved after splenectomy but required a decreased number of intermittent transfusions even post-splenectomy. This led to a significant iron overload for which he was treated with frequent phlebotomies, which facilitated our studies by providing us with large amounts of peripheral blood for the isolation of total reticulocyte RNA and genomic DNA. The propositus had a very atypical clinical presentation with a severe anisocytic and microcytic hemolytic anemia associated with a combined deficiency of spectrin and ankyrin and CNS involvement, including mental retardation. It was later determined that an ankyrin deficiency underscored the secondary deficiency of spectrin, a finding which represented the first seminal paradigm of a primary deficiency of ankyrin underlying a secondary spectrin deficiency manifested in HS.

Interpretation of the Clinical Phenotype in Terms of the Genetics of the HS Pedigree

It has been well documented that various genetic conditions segregate distinctly within given pedigrees; that is, the abnormal phenotype could be clearly distinguished from the normal one. In terms of clinical terms, however, some disorders have been known not to be expressed at all in individuals that were genetically predisposed; others, however, have tremendous variable expression in terms of clinical severity or age of onset, or both. The expression of an abnormal genotype may be influenced, and frequently modified, by other genetic loci or environmental factors. These differences in expression are particularly characteristic of autosomal dominant disorders, though by no means restricted to them,

often present complications in the diagnosis and the interpretation of pedigree data. In analyzing the clinical data of the given pedigree it was important to bear in mind the concepts of penetrance and expressivity, two significant characteristics of gene expression in autosomal dominant disorders with quite different meanings and implications (Thompson et al. 1991).

Penetrance refers to the concept of all-or-none expression of a mutant genotype; expressivity, on the other hand, is the extent to which a genetic defect is expressed. When the frequency of expression of a phenotype is below 100%, that is, when some of those who have the appropriate genotype completely fail to express it, the gene is then referred to as showing reduced penetrance. In terms of statistics, it would actually refer to the percentage of people with a particular genotype who are affected. On the other hand, when the expression of a genotype is different in people who are carriers of the same genotype, the phenotype is said to have variable expressivity. In such a case, a disorder could vary widely in its clinical expression even within a given kindred; in the event that more than one physiological system is affected by the disorder, such as blood and CNS, individual patients may show no clinical abnormality in one of the multiple systems affected. An example of this case is the well-known autosomal dominant condition known as the Marfan syndrome that is characterized by distinctive abnormalities involving three separate systems (Thompson et al. 1991).

Based on the evolving understanding of the clinical data and the family history, and despite the fact that individuals from the first generation were not available for evaluation, it was believed that the propositus could have been heterozygous for a dominant mutation showing reduced penetrance, variable expressivity, or both. He could have also been a compound heterozygote with one mutation at least responsible for the phenotype of non-anemic mild spherocytosis. The other mutation could be present in a regulatory domain of ankyrin or in another gene interacting with it. It is significant to mention that both the siblings of the propositus as well as his niece have clearly distinct clinical and genetic

phenotypes. His two siblings have mild HS and are considered subclinical. His niece on the other hand (III:2), although more severely anemic than her uncle (II:7) and her mother (II:5), is still of a milder clinical phenotype than the propositus, additionally lacking any sort of CNS involvement. It was conceivable that individual III:2 might have been a compound heterozygote having inherited one allele from her mildly affected, subclinical father and another one associated with the mild phenotype of HS present in the rest of the family, including her mother (II:4) and her uncle (II:3). Variable expressivity may explain the phenotypic features of individuals II:5, II:7, III:4, III:5, III:6, and IV:1. On the other hand, the report of normal hematological findings on the parents of the propositus, individual I:2 and I:3, may be explained by possible reduced penetrance, in which case they would carry the abnormal allele, but would not express it at the phenotypic level at all. Alternatively, the lack of abnormal hematologic findings in the propositus' parents may be due to the lack of sensitive diagnostic tests during their evaluation. Such tests might have revealed similar clinical phenotypes as with the rest of the affected family members, where variable expressivity of the abnormal allele may be implicated.

Erythrocyte ANK1 expression has been known not to be limited to red blood cells only. It has been reported to be highly expressed in the brain, especially the cerebellum, at a relatively late stage in the brain development, confined to a certain subset of neurons: Purkinje and granule cells in the cerebellum, most motor neurons of the spinal cord, and a small number of neurons in the forebrain. It has been, however, excluded from myelinated axons. Understanding the structure and the tissue- and developmental stage-specificity of ankyrin may prove to have important implications beyond the human blood. Particularly, in the proband in whom, along with the severe hematological findings, there had been clear CNS involvement.

Alternative mRNA Processing of Erythrocyte Ankyrin

The levels of specific gene products during development have been known to be regulated by activities relating to RNA production, maturation, transport, or stability (Leff et al. 1986). Complex transcriptional units could give rise to multiple protein polypeptides, and since nonprotein coding segments of mRNA may contain sequences instrumental to the regulation of protein products, this definition could be extended to transcription units that facilitated the production of multiple mature mRNA species. The production of multiple mRNA species from a single transcriptional unit may have involved usage of alternative signals for any of the steps of mRNA production or processing (transcript initiation and promoter signals, termination of transcription and 3' end formation, as well as mRNA splicing). Multiple mRNAs may arise from the use of multiple sites of initiation or polyadenylation, or from the use of alternative modes of exon splicing, or from any combination of these.

The mechanism(s) that may regulate the production of multiple species of ankyrin mRNA, or that may underlie the increase in the ankyrin transcript ratio that has been evident in the propositus, still remain unknown. In addition, the nature of the abnormality that underscores the selective decrease in the larger transcript of ankyrin manifested in the proband still remains to be elucidated. It was, however, clear that no major deletions or other rearrangements occurred in the ankyrin cDNA, and there was no evidence indicative of alternative mRNA processing in the ankyrin cDNA (ORF and UTRs).

A molecular abnormality occurring at the level of genomic DNA could provide an explanation for the selective deficiency in the larger ankyrin transcript. A similar phenomenon has been reported, whereby a nonsense mutation in the regulatory domain of ankyrin led to a marked and selective deficiency of the ankyrin isoform 2.1 and a normal content of the other isoform of ankyrin (2.2). It was suggested that as a result of that nucleotide substitution, alternative splicing within the regulatory domain led to retention of the respective codon in one isoform of ankyrin (2.1) and to the removal of the exon that

contained the same codon in the other isoform (2.2). That mutation could only be detected in genomic ankyrin DNA, and was absent from reticulocyte RNA, suggesting that the underlying mechanism of the selective ankyrin 2.1 deficit may have involved instability of the 2.1 mRNA which contained the mutation (Jarolim et al. 1995a). The lack of knowledge of the intron/exon boundaries of the ankyrin gene, along with the large size of the gene (>100 kb) and the number of known exons (40) would make the study of the genomic sequence of ankyrin, for such purposes, very difficult.

Search for Ankyrin cDNA Mutations

The absence of major deletion and any other rearrangements at the level of cDNA mandated the search for smaller deletions, single nucleotide substitutions, or both. The screening method of choice was SSCP analysis followed by confirmation of any potential DNA changes and their delineation by sequencing and ultimate distinction between nonspecific polymorphisms and disease-specific mutations by means of ASPCR. PCR-amplified cDNA from the propositus and unrelated normal controls was analyzed by SSCP. Two consistently abnormally migrating PCR products that were indicative of underlying DNA changes were identified. Their further analysis, which included subcloning and sequencing, did not reveal any change in their primary nucleotide sequencing. The failure to detect the presence of possible mutations could be related to the fact that the sensitivity of SSCP was optimally 80%. The technique with the highest possible sensitivity remained sequencing of the entire ankyrin cDNA.

Analysis of the $(AC)_n$ Repeat Polymorphism and Comparison of the Repeat Length in the Genomic DNA and cDNA

Analysis and comparison of the PCR products from genomic DNA and cDNA readily demonstrated the heterozygosity for this ankyrin AC repeat-length polymorphism in the proband (fig. 29 and 30). This data was indicative of the fact that both ankyrin alleles were transcribed normally, thereby establishing the fact that the transcripts were indeed present in sufficient amounts to consistently be assayed. These findings ruled out the possibility of

a null mutation, as was originally hypothesized (Hanspal et al. 1991), or a trace amount allele present in the propositus.

The mechanism(s) underlying the defect in the synthesis or stability of ankyrin leading to the decreased ankyrin mRNA content and the thalassemia-like membrane protein mutation remain unidentified. It is likely that, although the ankyrin mRNA is dramatically decreased, additional factors may have underscored the pathogenesis of HS in the proband. The initiation of synthesis of membrane skeleton proteins has been known to exhibit an asynchronous mode in which synthesis of spectrin, ankyrin and protein 4.1 is active well before that of band 3 protein. With the induction of band 3 synthesis the formation of a stable skeleton ensues with the assembly of the newly synthesized spectrin, ankyrin and band 4.1 proteins. During this process, band 3 becomes instrumental in the stability and assembly of the spectrin and ankyrin molecules on the membrane by recruiting and stabilizing the newly synthesized spectrin and ankyrin protein molecules on the membrane. The incorporation of the peripheral membrane proteins into the membrane skeleton as part of the process of terminal red blood cell maturation has been known to be modulated by enhanced recruitment and increased stability rather than transcriptional up regulation. On the other hand, spectrin and ankyrin that are localized in the cytosol would undergo rapid degradation irrespective of the stage of erythroid differentiation. The increased skeletal assembly of both spectrin and ankyrin during the terminal stages of the erythrocyte maturation, despite their reduced synthesis, is known to be related to the increased synthesis of band 3 and protein 4.1. It is, therefore, conceivable that a defect in the processing and / or transport machinery that involved band 3 and would result in failure to recruit and stabilize ankyrin onto the membrane could lead to rapid degradation of the ankyrin molecules that remained in the cytosolic environment (Lazarides and Woods 1989; Hanspal and Palek 1992; Hanspal et al. 1993).

FUTURE CONSIDERATIONS

Having provided evidence that the transcripts of both ankyrin alleles are readily detectable in comparable amounts in the propositus, the necessity of performing studies involving erythroid progenitor cells (BFU-E and CFU-E) in order to study a potential major defect in the transcription of erythroid ankyrin gene has been eliminated. The next step in the molecular characterization of the mutation that might explain the defect in the synthesis, assembly, or stability of ankyrin in the propositus would be the detection of mutation(s) at the genomic DNA level, a task which appears to be exceedingly difficult. The size of the gene encoding ankyrin is very large (>100 kb) with a large number of exons (~40), and the promoter regions have not been completely characterized. As a consequence, the hypothetical possibilities that could be tested to elucidate the nature of the molecular defect that underscores HS in the propositus are quite limited. These include commonly accepted mutations involving the translation initiation sequence, the polyadenylation signal that may result in inefficient polyadenylation and a concomitant mRNA instability. Additionally, large gene rearrangements could prevent transcription or result in unstable transcript. This possibility has been ruled out, both at the genomic DNA level as well as at the level of cDNA.

Our lack of knowledge of ankyrin remains impressive (Peters and Lux 1993). Questions about ankyrins increase faster than knowledge accumulates.

(1) A few ankyrins or many? Do a few ankyrins interact with many integral proteins or are there many ankyrins, each possibly reacting differently with accessory molecules? The knowledge available to date favors a limited number, although it raises questions concerning the ability of a few molecules to manage multiple integral protein interactions.

(2) Ankyrin specificity. Does the presence of one form of ankyrin in different tissues imply a similar function? Do all the cells that express erythroid spectrin concomitantly express Ank1?

(3) Regulation of ankyrin specificity. How do ankyrins regulate their specificity? By alternative splicing or post-translational modification (especially phosphorylation), or maybe both? Are the different "spliceoforms" of Ank1 distributed differently within cells, or are they bound to different ligands?

(4) Ankyrin distribution. Do different ankyrin molecules participate in membrane trafficking, or do they just anchor proteins once they get to the membrane? Are ankyrins found outside the plasma membrane, for example, in the nucleus?

(5) Ankyrin structure. What is the structure of ankyrin? Are the three domains physically separate? How does the regulatory domain relate to the other two domains? How are the repeats exactly organized? Are the amino acids in the first half of the repeats conserved because they participate directly in binding interactions?

(6) Physiologic importance of ankyrins. What are the consequences of disrupting specific ankyrin genes in mice? Are ankyrins present in more genetically malleable organisms such as Drosophila or yeast? Can one functionally disrupt individual binding sites by overexpression of single domains in tissue culture cells or transgenic mice? What diseases are associated with defects or deficiencies of human or mouse ankyrins? What are these defects, specifically, and what insight do they give us into ankyrin function?

SUMMARY

(1) Eleven potentially informative meioses for the study of HS have been identified in a family pedigree of varying degrees of inherited spherocytosis with or without hemolytic anemia.

(2) Based on the evolving understanding of the clinical data, the propositus (II:3) could have been heterozygous for a dominant mutation showing reduced penetrance and/or variable expressivity. He could have also been a compound heterozygote with one mutation at least responsible for the phenotype of nonanemic mild spherocytosis. The other mutation could be present in a regulatory domain of ankyrin or in another gene interacting with it.

(3) The two siblings of the propositus (II:5 and II:7), as well as his niece (III:2), clearly have distinct clinical and genetic phenotypes.

(4) Both II:5 and II:7 have mild HS and are considered subclinical. Individual III:2, although more severely anemic than both II:5 and II:7, is still of a milder clinical phenotype than the propositus lacking any CNS involvement. Genetically, III:2 could be a compound heterozygote having inherited one allele from her mildly affected father and another one associated with the phenotype of mild HS present in the rest of the family, including her mother (II:4).

(5) Variable expressivity could explain the phenotypic features of individuals II:5, II:7, III:4, III:5, III:6, and IV:1. On the other hand, the report of normal hematological findings in both parents of the propositus could be due to reduced penetrance. Alternatively, the normal findings might be explained by the lack of sensitive diagnostic tests during their evaluation.

(6) The abnormal ratio of the erythrocyte ankyrin transcripts was confirmed in the propositus. The nature of the abnormality that underscores this abnormal ratio appears not to be related to deletions and other rearrangements of the ankyrin cDNA. Additionally, there was no evidence indicative of alternative mRNA processing in the ankyrin cDNA (ORF and UTRs) occurring in the propositus. A molecular abnormality occurring at the level of genomic DNA could explain the abnormal transcript ratio and the selective deficiency of the larger ankyrin transcript.

(7) Screening of the ankyrin cDNA using SSCP analysis, followed by subcloning and sequencing of abnormally migrating fragments, did not reveal any changes in their primary nucleotide sequence. The failure to detect the presence of mutations could be related to the fact that the sensitivity of SSCP is not 100%.

(8) Analysis and comparison of an AC repeat polymorphism in the 3'-UTR of ankyrin, readily demonstrated in the propositus heterozygosity for this repeat. This provided evidence that the transcripts from both ankyrin alleles were readily detectable in comparable amounts. These findings ruled out the possibility of a null allele, as was originally hypothesized, or a trace amount allele present in the propositus, and have eliminated the necessity to perform nuclear run-on/run-off studies involving erythroid progenitors in order to study a major defect of human erythroid ankyrin (ANK1) transcription .

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