Spectrin Rouen ($\beta^{220-218}$), a Novel Shortened β-Chain Variant in a Kindred with Hereditary Elliptocytosis

Characterization of the Molecular Defect as Exon Skipping Due to a Splice Site Mutation

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Abstract

The molecular defect responsible for the shortened β-spectrin chain variant, spectrin Rouen, was identified by analysis of cDNA and genomic DNA of affected individuals after amplification by the polymerase chain reaction. Peripheral blood reticulocyte RNA was transcribed into cDNA and amplified using primers corresponding to the 3′ end of β-spectrin cDNA. Agarose gel electrophoresis of cDNA amplification products from affected individuals revealed the expected band of 391 bp as well as a shortened band of 341 bp. Nucleotide sequencing of the shortened cDNA amplification product revealed that the sequences corresponding to the penultimate exon of the β-spectrin gene (exon Y) were absent. This result was confirmed by hybridization of a Southern blot of amplification products with a labeled probe specific for exon Y. Nucleotide sequencing of the proband's amplified genomic DNA corresponding to this region of the β-spectrin gene revealed a mutation in the 5′ donor consensus splice site of the intron downstream of the Y exon, TGG/GTGAGT to TGG/GTTAGT, in one allele. We postulate that this mutation leads to the splicing out or skipping of exon Y, thus producing a shortened β-spectrin chain. To our knowledge, this is the first documented example of exon skipping as the cause of a shortened β-spectrin chain in a case of hereditary elliptocytosis. The exon skip results in the loss of the 17 amino acids of exon Y and creates a frameshift with the synthesis of 33 novel amino acids prior to premature chain termination 14 residues upstream of the normal carboxy terminus of the β-spectrin chain, giving a mutant β-spectrin chain that is 31 amino acids shorter than the normal chain. (*J. Clin. Invest.* 1991. 88:76–81.) Key words: hemolytic anemia • erythrocyte membrane skeleton • DNA sequence • polymerase chain reaction • phosphorylation sites

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Introduction

Spectrin Rouen is a novel shortened β-spectrin chain variant that is associated with hereditary elliptocytosis (HE)[1] in a French kindred (1).

A number of different possible mutations could result in a shortened or truncated polypeptide chain. These include partial gene deletions, nonsense mutations and frameshift mutations resulting in premature chain termination during mRNA translation, and mutations resulting in alternative splicing or exon skipping owing to creation of novel or alterations of normal splice consensus sequences.

To determine the molecular basis for spectrin Rouen, we carried out a structural analysis of the cDNA and genomic DNA corresponding to the 3′ end of the β-spectrin gene of affected individuals. The mutation was suspected to reside in this region of the β-spectrin gene because the mutant Rouen spectrin β' chain is nonreactive with a monoclonal antibody that recognizes an epitope at the carboxy terminus of the normal β-spectrin chain. In addition, the carboxy terminus is the region of the normal β-spectrin chain that is phosphorylated and involved in spectrin dimer self-association. Spectrin Rouen is associated with decreased phosphorylation as well as defective spectrin dimer self-association, resulting in HE.

Analysis of the β-spectrin cDNA of affected individuals revealed evidence of exon skipping with absence of sequences encoded by the penultimate exon of the β-spectrin gene. Sequence analysis of the corresponding genomic DNA demonstrated a point mutation in the 5′ (donor) consensus splice sequence downstream of the skipped exon.

Methods

Synthesis and amplification of reticulocyte β-spectrin cDNA. Human reticulocyte RNA was prepared from peripheral blood as described (2). 0.5 μg of total RNA was transcribed into single-stranded cDNA by incubation for 30 min at 42°C in a 20-μl reaction mixture containing 75 mM KCl, 50 mM Tris-HCl (pH 8.3), 3 mM $MgCl_2$, 10 mM DTT, 1 mM dNTPs, 0.1 μg of oligo-dT, 2 μg of bovine serum albumin, 20 U RNAsin (Promega Corp., Madison, WI), and 100 U of M-MLV reverse transcriptase (Gibco BRL, Gaithersburg, MD). The sample was then heated for 2 min at 100°C and quickly cooled on ice. The cDNA was then amplified by the polymerase chain reaction (PCR) (3, 4) in a 100-μl reaction mixture containing 50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 0.1% gelatin, 50 pmol of each primer, 0.2 mM dNTPs, and 2 U of *Taq* polymerase. 30 cycles of amplification were carried out in an automated DNA thermal cycler (Perkin-Elmer Cetus, Norwalk,

1. *Abbreviations used in this paper:* dNTP, deoxynucleoside triphosphate; HE, hereditary elliptocytosis; M-MLV, Moloney murine leukemia virus; PCR, polymerase chain reaction.

CT). The cycles consisted of 60 s at 94°C, 90 s at 45°C, and 120 s at 72°C. The first cycle was initiated with a 5-min denaturation step. After the last cycle, the samples were incubated at 72°C for 7 min to ensure the completion of the last extension step. Amplified DNA products were analyzed by electrophoresis in 2% agarose gels. The sequences of the upstream and downstream primers utilized were:

5′-CGT(GGATCC)GTGGCTGAGGCGTGGCTGATTGC-3′

and

5′-CGT(AAGCTT)CTGCGCAGCTCATCTCGCCT-3′,

respectively. Their 5′ extremities contain recognition sequences (shown in parentheses) for the restriction enzymes *Bam*HI and *Hind*III, respectively. They are designed to anneal to target sequences located approximately 400 bp apart at the 3′ end of the coding region of β-spectrin cDNA.

Southern blot of amplified cDNA. Amplified cDNA products were fractionated by electrophoresis in a 2% agarose gel and transferred to a nylon membrane (BioTrace, Gelman Sciences, Inc., Ann Arbor, MI) by Southern blotting under alkaline conditions (6). The filter was hybridized by standard methods to a ^{32}P-labeled 0.6 kb *Xho*I/*Eco*RI β-spectrin cDNA fragment corresponding to the 3′ end of the β-spectrin mRNA. The hybridized filter was exposed to x-ray film overnight at −80°C with a single intensifying screen. The filter was then stripped of the probe by boiling in water for 5 min and rehybridized to a labeled genomic fragment encompassing only the penultimate exon (exon Y) of the β-spectrin gene (7). The hybridized filter was then exposed to x-ray film overnight as above.

Direct sequencing of amplified cDNAs. The PCR products were fractionated by electrophoresis in a 2% agarose gel. Two bands of 391 and 341 bp were visualized by ethidium bromide staining and each band was excised from the gel. DNA was recovered from the agarose by freezing of the gel slice in liquid nitrogen followed by thawing and centrifugation. The DNA in the supernatant was denatured in 0.4 M NaOH, precipitated by centrifugation after addition of sodium acetate and ethanol, then dissolved in water. DNA sequencing was performed using the T7 DNA polymerase sequencing kit (Pharmacia, St. Quentin en Yvelines, France) according to the manufacturer's instructions. In a typical experiment, ~ 300 ng of denatured DNA was annealed to 100 ng of an internal primer located 5′ to the downstream primer used for the PCR. The sequence of the internal primer was 5′-CACCTGGGCTGAGCTAGTAG-3′.

Subcloning and sequencing of amplified cDNAs. Amplified cDNA was purified by sequential extractions with phenol/chloroform and chloroform, and precipitated with ethanol. The DNA precipitate was then resuspended in 20 μl of restriction enzyme buffer, digested sequentially with *Bam*HI and *Hind*III, and fractionated by electrophoresis in a 2% NuSieve GTG (FMC BioProducts, Rockland, ME) agarose gel. The PCR products were excised from the gel and DNA recovered by phenol extraction at 65°C. The fragments were then subcloned in plasmid pGEM3Z (Promega Corp.), which had been first digested with *Bam*HI and *Hind*III. After transformation in Epicurian Coli XL1-BLUE competent cells (Stratagene, LaJolla, CA), recombinant clones were isolated and their DNA purified. Subclones containing the amplified DNA were identified by digestion of the plasmid DNA with *Hind*III and *Eco*RI and electrophoresis in 2% agarose gels. Sequencing of cDNA inserts was carried out by the dideoxy sequencing method of Sanger et al. (8) with T7 DNA polymerase (Sequenase, U. S. Biochemical Corp., Cleveland, OH) using Sp6 and T7 promoter primers.

Genomic DNA analysis. Genomic DNA was prepared from peripheral blood leukocytes by standard methods. Approximately 0.2–1 μg was used in each PCR amplification reaction. The cycles consisted of 94°C for 1.4 min, 55°C for 2 min, and 72°C for 3 min, for 30 cycles, and the primers used were primer A (5′-GTGGCTGAGGCGTGGCTGATTGC-3′) and primer B (5′-CACCTGGGCTGAGCTAGTAG-3′) located in exons X and Z, respectively, of the β-spectrin gene, and separated by approximately 1.2 kb in genomic DNA (7). Amplified PCR products were fractionated by electrophoresis in 1% agarose gels. Slices containing the desired products were excised and the DNA eluted electrophoretically in an Elutrap apparatus (Schleicher & Schuell, Inc., Keene, NH). The DNA fragments were then subcloned into plasmid pGEM4Z (Promega Corp.). Isolation of recombinant plasmids and sequencing of the inserts were performed as described above.

Allele-specific oligonucleotide hybridization. PCR amplified genomic DNA was transferred onto nylon membranes (Nytran, Schleicher & Schuell, Inc.) by a slot blot apparatus (Manifold, Schleicher & Schuell, Inc.). Allele-specific oligonucleotide hybridization was performed as described (3) except that the final wash condition was at 60°C for 10 min. The probes used in the hybridization were oligonucleotides 5′-CCACCACTAACCCAGTCTCC-3′ and 5′-CCACCACTCACCCAGTCTCC-3′, which correspond to the normal and mutant alleles, respectively.

Results

cDNA analysis. In order to identify the molecular defect that causes the shortening of the β-spectrin chain associated with spectrin Rouen, we analysed the structure of β-spectrin gene transcripts from the proband. cDNA corresponding to the 3′ end of β-spectrin mRNA was synthesized from peripheral blood reticulocyte RNA of the proband and amplified by PCR using primers corresponding to sequences bracketing the last three exons (X, Y, Z) of the β-spectrin gene (7).

Agarose gel electrophoresis of the PCR products revealed the presence of cDNA fragments of two different lengths (Fig. 1 *A*). One species was 391 bp in length, consistent with the expected size of the normal PCR product (5). The other species was 341 bp in length. The appearance of a shorter cDNA species suggested that it might encode the shortened β chain of

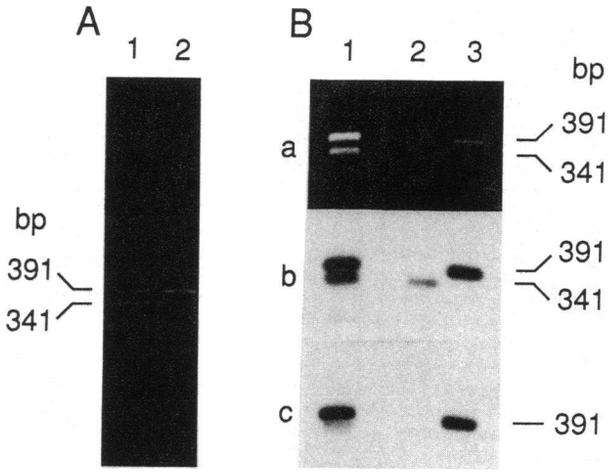

Figure 1. Products obtained by PCR amplification of reticulocyte β-spectrin cDNA. (*A*) Agarose gel stained with ethidium bromide. Lane *1:* cDNA of a subject with spectrin Rouen showing a shortened 341-bp product in addition to the normal 391-bp product. Lane *2:* cDNA of a normal control subject. (*B*) Panel *a:* Agarose gel stained with ethidium bromide. Lane *1:* amplified cDNA of a subject with spectrin Rouen. lanes *2* and *3:* cDNA products from the lane *1* sample after purification by gel elution. Panel *b:* Southern blot of the gel hybridized to a ^{32}P-labeled β-spectrin cDNA probe. Panel *c:* Rehybridization of the blot to a genomic probe including only exon Y of the β-spectrin gene.

spectrin Rouen and that it might have arisen from aberrant mRNA splicing.

To test this possibility, we carried out direct DNA sequence analysis of the two separate cDNA products. The DNA sequence of the longer PCR product was normal as expected, but the sequence of the shorter product revealed that the sequence encoded by the penultimate exon (exon Y) of the β-spectrin gene was absent from the cDNA. The sequence encoded by exon X was spliced directly to that encoded by exon Z (data not shown). The same results were obtained by DNA sequence analysis of the same PCR products subcloned into plasmid vectors (Fig. 2).

To confirm this finding, the gel-fractionated PCR products were transferred to a nylon membrane by Southern blotting and hybridized to a β-spectrin cDNA probe. Both cDNA products gave positive hybridization signals, confirming that they were authentic β-spectrin cDNA products (Fig. 1 *B*, panel *b*). The filter was then stripped of the cDNA probe and rehybridized with a genomic DNA fragment of the β-spectrin gene that contained only exon Y. Only the cDNA species of the normal size (391 bp) gave a positive hybridization signal (Fig. 1 *B*, panel *c*) indicating that the sequence corresponding to exon Y was not present in the shortened (341 bp) cDNA fragment. These results confirmed that spectrin Rouen is associated with a shortened β-spectrin mRNA species that does not contain the sequence encoded by exon Y of the β-spectrin gene. This abnormal mRNA species most likely arose from exon skipping due to an aberrant mRNA splicing event.

Fig. 3 shows the expected amino acid sequence of the truncated β-spectrin chain of spectrin Rouen as predicted from the sequence of the shortened cDNA. The skipping of exon Y leads to the deletion of the amino acid sequence encoded by this exon, but also results in a frameshift mutation in the translation of the sequence encoded by exon Z. A termination codon is encountered 99 bp downstream in the new reading frame. As a result, the carboxyl-terminal 64 amino acid residues of the normal β-spectrin chain, beginning at codon 2074, are replaced by a variant carboxy-terminus 33 residues in length. There is thus a net loss of 31 amino acid residues in the variant β-spectrin chain, an amount that corresponds to ~ 3 kD in molecular mass and agrees with the ~2-kD shortening of the β′ chain of spectrin Rouen, as estimated by SDS-polyacrylamide gel electrophoresis.

Genomic DNA analysis. To determine the basis for the aberrant splicing event, we analyzed the sequence of the 3′ end of the proband's β-spectrin gene by PCR. Genomic DNA corresponding to the 3′ end of the gene was amplified, subcloned into plasmid vectors, and sequenced. The sequence of the entire coding region of exon Y as well as the acceptor splice site preceding it was normal in all eight subclones analyzed. However, we found a G to T transversion at the third position of the donor splice consensus sequence downstream of exon Y in four out of the eight subclones: TGG/GTGAGT to TGG/GTTAGT (Fig. 4). This result indicated that a mutation in the donor splice site of the intron downstream of exon Y was the likely cause of the exon skipping.

We correlated the presence of this mutation with the clinical and biochemical phenotypes in this kindred by allele-specific oligonucleotide hybridization (Fig. 5). The proband and his affected mother have both the normal and mutant allele (lanes *1* and *2*). DNA from a normal control and two patients with other shortened β-spectrin chain variants, spectrin Nice (9) and spectrin LePuy (10), hybridized only to the normal probe (lanes *3*, *4*, and *5*, respectively). These results indicate that the identified mutation is co-inherited with spectrin Rouen and is therefore the likely cause of the disorder.

Discussion

The identification of the molecular defect associated with the truncated β chain of spectrin Rouen helps to address several basic issues about the structure–function relationships of spectrin. Specifically, it provides information regarding the region of the β-spectrin chain that is important for spectrin dimer self-association as well as an indication of potential sites of phosphorylation of the chain. In addition, the results shed light on the general process of splicing and exon skipping.

Previous functional studies of spectrin have shown that the carboxy terminus of the β-spectrin chain participates in the interaction between spectrin dimers (reviewed in references 11–13). However, the exact mechanism of the interaction is unknown. In spectrin Rouen, the substitution of the carboxy-terminal 64 residues of the normal β-spectrin chain by a variant carboxy terminus of 33 residues interferes with spectrin dimer self-association, with the resulting phenotype of HE. Another consequence of the spectrin Rouen mutation must be a change in the conformation of the amino terminus of the α-spectrin chain (1) because it manifests an enhanced susceptibility to proteolysis, resulting in increased amounts of the αI/74-kD fragment after limited tryptic digestion (M.-C. Lecomte, A.

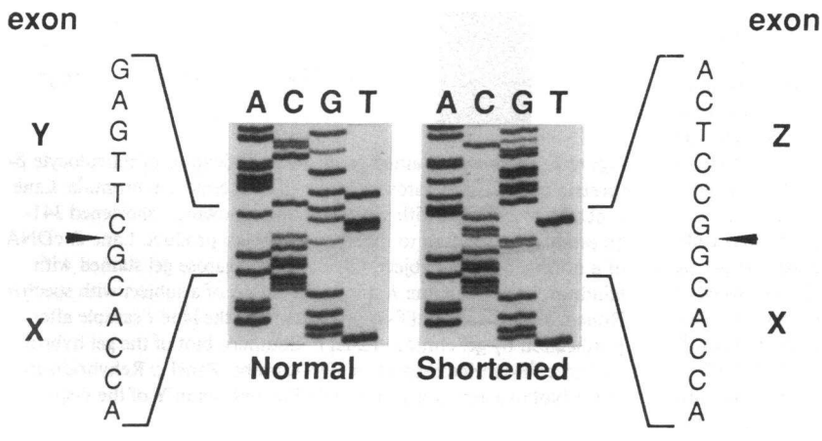

Figure 2. DNA sequence of subcloned β-spectrin cDNA products from subject with spectrin Rouen. Nucleotide sequence of the normal (*left*) and shortened (*right*) PCR products, corresponding to sequences encoded by exons X and Y (*left*) and by exons X and Z (*right*) of the β-spectrin gene. These sequence data are available from EMBL/GenBank/DDBJ under accession number J05500.

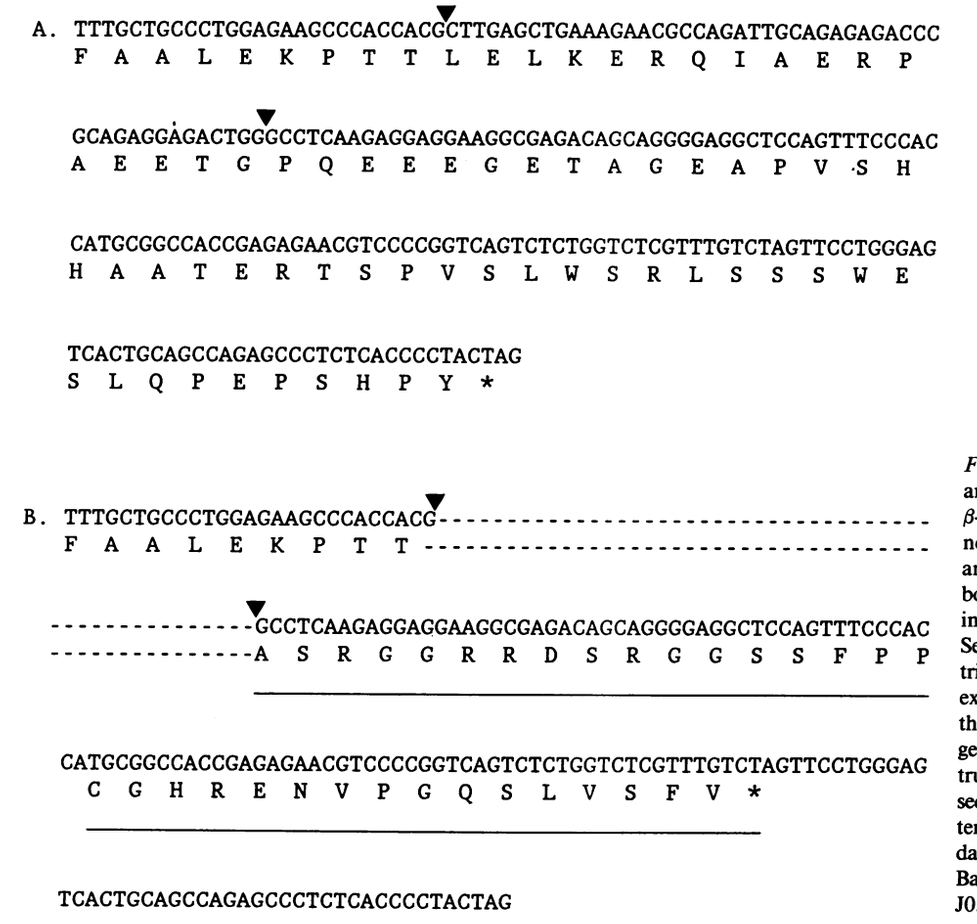

Figure 3. DNA sequence and translated amino acid sequence of the 3′ end of β-spectrin cDNA. (*A*) Sequence of the normal β-spectrin cDNA and translated amino acids. The location of exon boundaries in the genomic DNA are indicated by the inverted triangles. (*B*) Sequence of the mutant Rouen β-spectrin cDNA: the nucleotides encoded by exon Y are missing (*dashed line*) and the resulting frameshift results in the generation of a variant (*underlined*) and truncated carboxy-terminal amino acid sequence of the Rouen β chain. *Chain termination codon. These sequence data are available from EMBL/GenBank/DDBJ under accession number J05500.

Lahary, J. P. Vannier, H. Gautero, C. Galand, O. Bournier, M. Montconduit, P. Thron, P. Boivin, and D. Dhermy, personal communication). Abnormal spectrin dimer self-association and an increase in the amount of the αI/74-kD fragment were also observed in a family with HE presumably due to an amino acid substitution at position 2053 in the carboxy-terminal region of the β-spectrin chain (7).

A model has been developed (7) in which the ends of the α- and β-spectrin chains are proposed to interact with one another to form a triple α-helical structure similiar to that hypothesized for the individual 106 amino acid repeats of the α- and β-spectrin chains (14). Disruption of this proposed hybrid structure by a mutation in the carboxy terminus of the β chain could theoretically perturb the conformation and other properties of the amino terminus of the α chain. Thus, the amino terminus of the α chain might be exposed to enhanced proteolysis, giving

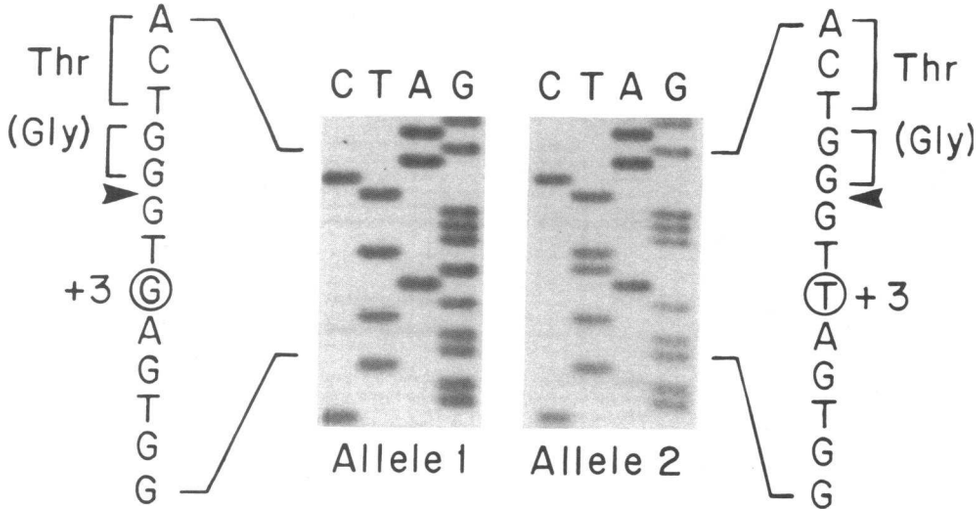

Figure 4. Mutation in a 5′ donor splice site of the Rouen β-spectrin gene. DNA sequence of the 5′ donor splice site of the intron downstream of exon Y showing a G to T substitution at the third position of the consensus sequence (*circled*) in the mutant allele (*right*), compared to the normal allele (*left*). The arrowheads indicate exon/intron boundaries. These sequence data are available from EMBL/GenBank/DDBJ under accession number J05500.

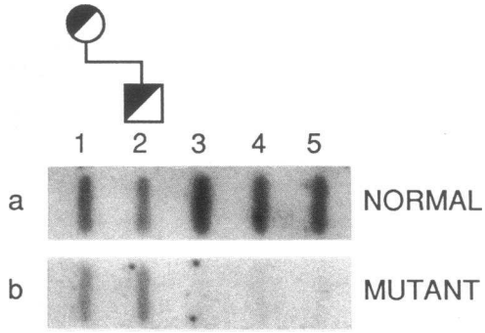

Figure 5. Allele-specific oligonucleotide hybridization. Slot blots of amplified genomic DNAs hybridized with ^{32}P-labeled oligonucleotide probes corresponding to the 5′ donor splice site downstream of exon Y of either the normal β-spectrin gene (*a*) or the mutant (*b*). Only the proband and his affected mother carry the mutant allele; lane *1*, mother of the proband; lane *2*, proband with spectrin Rouen; lane *3*, a normal control subject; lane *4*, a patient with spectrin LePuy; lane *5*, a patient with spectrin Nice.

rise to the increased amount of the αI/74-kD fragment seen in spectrin Rouen. The model explains the various biochemical effects seen in this disorder.

In contrast to other known examples of shortened β-spectrin chain variants, spectrin Rouen maintains partial ability to be phosphorylated (1). Harris and Lux (15) have previously shown that there are at least four phosphorylation sites clustered in a carboxy-terminal 9-kD fragment of the β-spectrin chain. Analysis of the cDNA sequence corresponding to the carboxy terminus of the β-spectrin chain reveals the sequence LSSSW, at codons 2123–2127, that is consistent with a casein kinase consensus sequence and may constitute one of these phosphorylation sites (16). Mische and Morrow (17) found that the total endogenous phosphate in spectrin dimers is 4.4 phosphates per mole of spectrin and demonstrated that multiple kinases phosphorylate spectrin, including cAMP-independent (casein kinase), cAMP-dependent and calcium/calmodulin kinases. The translated amino acid sequence of the truncated β chain in spectrin Rouen is normal up to codon 2074 (Fig. 3). Since the β chain of spectrin Rouen is at least partially phosporylated (1), this result suggests that at least one phosphorylation site of β-spectrin is located upstream of residue 2074. Alternatively, the phosphorylation of the Rouen β-spectrin chain could involve the novel carboxy-terminal segment of the mutant β-spectrin chain.

Spectrin Rouen is the first documented example of an HE-associated spectrin variant that is caused by a splicing defect and this defect results in the loss of one complete exon in the mutant mRNA, i.e., exon skipping. PCR analysis of reticulocyte cDNA has also been recently used to demonstrate the presence of shortened mRNA species in HE associated with shortened variants of protein 4.1 and glycophorin C, presumably because of exon skipping or recombination events (18, 19). There are several examples of exon skipping as the cause of genetic disease in various gene systems (references 20 and 21, and references therein). In all of these examples of exon skipping, the mutation is located at the 5′ (or donor) splice site of the intron immediately following (i.e., 3′ to) the skipped exon. Therefore, it was not unexpected to find a mutation in the 5′ splice consensus sequence of the intron immediately downstream of the skipped exon (exon Y) of the Rouen β-spectrin gene.

The mutation found in the Rouen β-spectrin gene occurs at the third position of the affected donor splice site consensus sequence. This is an unusual location for a splicing mutation. For instance, in the β-thalassemia syndromes, splice site mutations have only been found to involve positions −3, −1, 1, 2, 5 and 6 of the consensus sequence (22). Position 3 of the normal 5′ donor splice consensus sequences in humans is almost always a purine (A or G) (96% of sites): A (56%) and G (40%) (23). Therefore, an A to C transversion (purine to pyrimidine) constitutes a dramatic change from normal and might be expected to result in an aberrant splicing event. Until very recently, no other naturally occurring exon skipping mutation was shown to involve position 3 of the donor consensus splice site. The one other example consists of a G to A substitution at position 3 of the donor site of an ornithine transcarbamylase gene intron of an individual with ornithine transcarbamylase deficiency (21). Interestingly, the affected individual had a null phenotype, i.e., total absence of ornithine transcarbamylase and therefore total absence of normal splicing of the affected exon.

Paradoxically, however, a G to A mutation, produced by in vitro mutagenesis, at position 3 of IVS 2 of a rabbit β globin gene, had no deleterious effect on splicing following gene transfer in tissue culture cells (24). An A to T transversion produced by in vitro mutagenesis at the third position of an intron of the adenovirus 2 E1A gene totally prevented RNA splicing at the corresponding 5′ splice site (25). These observations support the proposal that the mutation we have described is the underlying defect responsible for exon skipping in spectrin Rouen.

What remains unresolved is whether the splicing defect of the Rouen β-spectrin gene is total or partial. The low level of expression of the mutant protein (< 10% of total β spectrin in heterozygotes [1]) suggests that the splicing defect may be partial and that the mutant gene is a plus rather than a null allele. However, the low level of expression of the mutant chain may be related to instability of the mutant mRNA (or protein) rather than due to the quantitative level of abnormal splicing of transcripts of the mutant allele. Unfortunately, the amount of the mutant versus the normal cDNA product obtained after PCR amplification cannot be used as an accurate measure of the steady state level of mutant versus normal mRNA because of nonlinearity of the PCR technique. Although the PCR amplifications of reticulocyte cDNA of a spectrin Rouen carrier shown in Fig. 1 yielded substantial amounts of the mutant product relative to the normal product, other amplifications yielded much lower relative amounts of the mutant product (data not shown). Studies of splicing of transcripts derived from the mutant allele in in vitro systems and/or after gene transfer in tissue culture cells should help to resolve this question.

Note added in proof. After submission of this manuscript, an article was published reporting the molecular basis of a different truncated β-spectrin chain due to exon skipping (Yoon, S.-H., Y. Huilan, S. Eber, and J. T. Prchal. 1991. Molecular defect of truncated β-spectrin associated with hereditary elliptocytosis. β-spectrin Göttingen. *J. Biol. Chem.* 266:8490–8494).

Acknowledgments

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