A Novel Mobile Element Inserted in the α Spectrin Gene: Spectrin Dayton

A Truncated α Spectrin Associated with Hereditary Elliptocytosis

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Abstract

Nonviral retrotransposons, retropseudogenes, and short interspersed nuclear elements (SINEs) are mobile DNA segments capable of transposition to new genomic locations, where they may alter gene expression. De novo integration into specific genes has been described in both germ and somatic cells. We report a family with hereditary elliptocytosis and pyropoikilocytosis associated with a truncated α-spectrin protein. We present the biochemical characteristics of this abnormal protein and show that the α-spectrin gene is disrupted by a mobile element resulting in exon skipping. This element causes duplication of the insertion site and is terminated by a long poly-A tail downstream of multiple consensus polyadenylation signals. Southern blot analysis of human genomic DNA, using this element as probe, reveals one to three copies per individual. This element has no homology to any previously reported sequence and therefore appears to be a member of a novel family of mobile elements. (*J. Clin. Invest.* 1994. 94:643–648.) Key words: mobile elements • retrotransposons • exon skipping • hereditary elliptocytosis • α spectrin

Introduction

Mobile DNA elements have been classified according to their primary structural organization which is believed to reflect the mechanism by which they are generated (1–4). Nonviral Retrotransposons, retropseudogenes, and short interspersed nuclear elements (SINEs),[1] are mobile DNA elements thought to be generated by reverse transcription of an mRNA intermediate and randomly reinserted in the genome where they may alter gene expression (5–9). This model is based on the characteristic primary structure of mobile elements, which includes a variable size poly-A tail preceded by one or multiple polyadenylation signals, direct flanking repeats representing duplication of the insertion site, and the absence of intervening sequences.

Human long interspersed nuclear elements (LINE-1), or L1Hs, constitute the only known family of human nonviral retrotransposons (for review see references 1 and 4). They are long, highly repeated DNA segments (10^4 to 10^5 copies), most of them truncated at the 5′ end, and have, as a group, a high degree of sequence homology (10–12). From the isolated full length elements, a consensus sequence has been established which has two large open reading frames, one of which encodes a reverse transcriptase (13–15). Retropseudogenes, on the other hand, are usually nonfunctional genomic segments, structurally similar to specific functional genes but lacking intervening sequences and dispersed to distant locations from that of the original gene which is fixed (for review see reference 3). SINEs are highly repeated elements, ~ 70–300 bp in length, present at a high copy number (10^5 to 10^6) in the genome (1, 2). With the exception of the human Alu family members, which can be viewed as retropseudogenes derived from 7SL RNA (16), SINEs have a more complex primary structure consisting of a region homologous to a tRNA, and a tRNA unrelated region (17–19) which contains two conserved motifs homologous to retroviral sequences. Therefore, the model suggested for the generation of SINEs differs from the one proposed for retropseudogenes and justifies their classification as a separate group (19).

De novo integration of mobile elements into specific genes has been described in both germ and somatic cells (5–9). We report here the disruption of the α-spectrin (Sp) gene by a novel mobile element, occurring in a family with hereditary elliptocytosis (HE) and pyropoikilocytosis (HPP), a common hemolytic anemia resulting from a defect of the red cell skeleton (for review see reference 20). Mutations of spectrin, the major red cell skeletal protein, have been reported in many patients with HE and HPP. This protein is composed of two chains, α and β, consisting of 20 and 17 homologous 106 amino acid repeat segments, respectively, folded in a triple helical structure (21). α and β spectrin are intertwined in an antiparallel manner to form a heterodimer (22). Spectrin heterodimers associate at their head region (23–25) forming tetramers or, occasionally, higher order oligomers. We describe here a mobile element inserted within the α-spectrin gene, causing in-frame deletion of exon 5, leading to a truncated protein, and show that this element belongs to a novel family of mobile elements.

Methods

Patients. The pedigree of the family under study is shown in Fig. 1. It consists of three generations. Subject I. 1 is asymptomatic and presents

Part of this work was presented at the 34th Annual Meeting of The American Society of Hematology, Anaheim, CA, December 1992.

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Received for publication 18 October 1993 and in revised form 25 March 1994.

1. *Abbreviations used in this paper:* HE, hereditary elliptocytosis; HPP, hereditary pyropoikilocytosis; LINE-1, long interspersed nuclear elements; SINEs, short interspersed nuclear elements; Sp, spectrin; SpD, spectrin dimer; Spt, spectrin tetramer.

J. Clin. Invest.

0021-9738/94/08/0643/06 $2.00
Volume 94, August 1994, 643–648

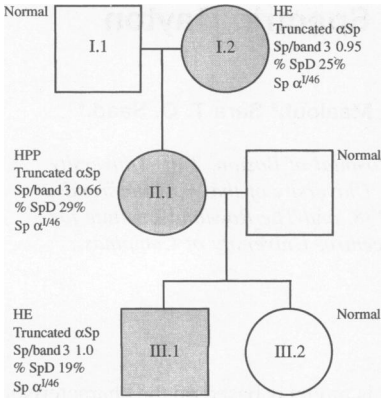

Figure 1. Pedigree of the family with hereditary elliptocytosis and pyropoikilocytosis. The clinical features are detailed in the method section.

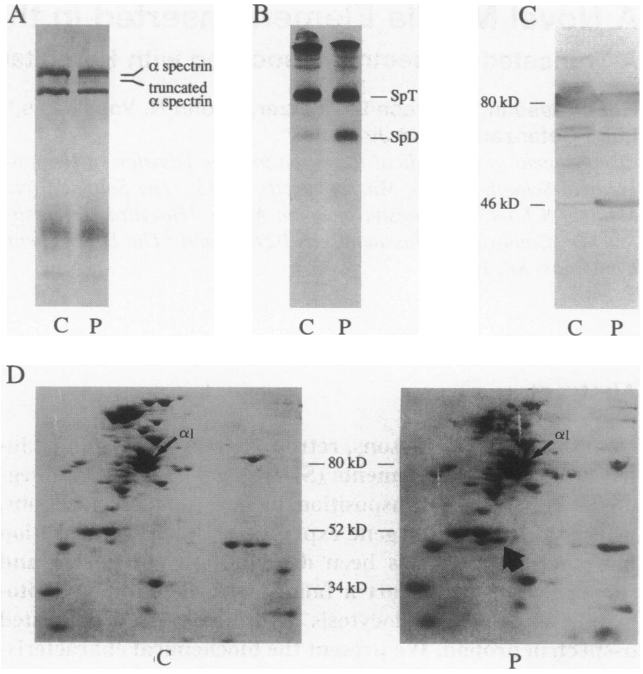

Figure 2. Representative biochemical analysis of the red cell membrane proteins in one of the affected members of the family under study (II.1). (*A*) SDS-PAGE analysis (12% Laemmli gel) of the red cell membrane proteins of a control subject (*C*) and one patient (*P*) revealing the presence of an extra band migrating below the α-spectrin band in the patient; (*B*) Electrophoresis under non denaturing conditions of spectrin extracted from the membrane at 4°C, showing an increase in the spectrin dimer to tetramer ratio in the patient (*P*) (29% SpD) compared to the control (*C*) (< 10% SpD); (*C*) Western blot analysis of spectrin tryptic fragments separated on a Laemmli gel, using an antibody directed against the 80-kD peptide of α spectrin. Note the increase in the 46-kD peptide in the patient's sample (*P*) compared with the control sample (*C*); (*D*) Two-dimensional isoelectric focusing/SDS-PAGE (10% acrylamide) of spectrin tryptic fragments showing the abnormal 46-kD peptide in the patient's sample (*large arrow*).

no detectable abnormalities. Patient I.2 has a mild compensated hemolytic anemia with a hematocrit of 35%, a reticulocyte count of 8.5%, and elliptocytes on the peripheral blood smear. Patient II.1 was severely anemic until age 9 when she underwent splenectomy. Since that date she has remained asymptomatic with a hematocrit of 36% and a reticulocyte count of 1.5%. Her peripheral blood smear shows numerous elliptocytes, poikilocytes, microspherocytes, and fragments. We have previously reported that this subject has two genetic defects of α spectrin: (*a*) one allele carrying the elliptocytogenic spectrin mutation which is the subject of this paper, and (*b*) the second allele carrying a synthetic defect resulting in spectrin under-production (26). Patient III.1 has a mild compensated hemolytic anemia with elliptocytosis and a hematocrit of 38% with 5.1% reticulocytes while subject III.2 has no detectable abnormalities.

Erythrocyte membrane protein analysis. The methods used for analysis of the erythrocyte membrane proteins have been described previously and include (*a*) erythrocyte membrane preparation (27, 28); (*b*) analysis of the red cell membrane proteins by SDS-PAGE (3.5 to 17% gradient Fairbanks and 12% Laemmli polyacrylamide gels) (29); (*c*) spectrin extraction at 4°C (27) and relative determination of spectrin dimers (SpD) and spectrin tetramers (SpT) by nondenaturing gel electrophoresis (30); (*d*) limited tryptic digestion of spectrin (31) followed by separation of the resulting fragments by SDS-PAGE (10% acrylamide) (29) as well as two-dimensional (2D) isoelectric focusing/SDS-PAGE (31); (*e*) immunoblotting using a polyclonal antibody raised against α spectrin, β spectrin, and ankyrin (27); and (*f*) direct amino acid sequencing of the abnormal 46-kD fragment eluted from the 2D gel (32).

PCR amplification of reticulocyte α-spectrin cDNA. 5 μg of total RNA isolated from reticulocytes (33) were reverse transcribed using random primers. The cDNA corresponding to exons 1 to 6 of α spectrin was amplified by the polymerase chain reaction (PCR) using primers 144 (5′-GATTGTGTATGGCTGATGGG-3′) and 225 (5′-TTTGCTTAGACTGAATTAAG-3′) shown in Fig. 3, in a 100-μl reaction mixture containing 50 mM KCl, 10 mM Tris-HCl, 2 mM $MgCl_2$, 0.1% gelatin, 100 pmol of each primer, 0.2 mM dNTP, and 2U of Taq polymerase. Each cycle consisted of 1 min at 95°C, 1 min at 50°C, and 2 min at 72°C. The fragments resulting from this amplification were fractionated on a 1% agarose gel and stained with ethidium bromide.

Southern analysis of genomic DNA. Probes were labeled with ^{32}P dCTP by random priming (Megaprime DNA labeling system, Amersham, UK). Genomic DNA was isolated from peripheral blood mononuclear cells as described previously (34). 10 μg of genomic DNA were digested with restriction enzymes. The resulting fragments were fractionated on a 0.7% agarose gel, transferred to a nylon membrane (Zetaprobe; Bio Rad laboratories, Inc., Richmond, CA) by nonalkaline Southern blotting and hybridized to the probe as described (35).

PCR amplification of a spectrin genomic DNA. Genomic DNA was isolated from peripheral blood leukocytes as described previously (34). Optimal amplification was obtained in a 100-μl reaction mixture containing 50 mM KCl, 10 mM Tris-HCl, 4 mM $MgCl_2$, 0.1% gelatin and 100 pmol of primers 264 (5′-GTGCTGACATCTTAGAGTGG-3′) and 268 (5′-TTAAGGGTAGGTCAGGATGG-3′) shown in Fig. 5. A 40 cycle amplification was carried out, each cycle consisting of 1 min at 95°C, 1 min at 50°C, and 10 min at 72°C.

Subcloning and sequencing of amplified DNA. DNA fragments generated by PCR amplification were fractionated on a 1% agarose gel, purified using the Geneclean system (BIO 101, Natick, MA) and subcloned into the pCRII cloning vector (Invitrogen Corp., San Diego, CA). After transformation in competent *E. coli* cells, the recombinant DNA clones were sequenced by the dideoxynucleotide sequencing method of Sanger et al. (36).

Computer homology search for DNA sequences. The query sequence was analyzed in both orientations using the BLAST (37) and FASTA (38) programs. The nucleotide sequence databases searched included GenBank 78.0, and EMBL Library 35.0. In addition, all 6 open reading frames were analyzed against the Brookhaven Protein Data Bank (June 1993 release), SWISS-PROT 26.0, PIR 37.0 (complete), and Genpept-CDS translations from GenBank 78.0.

Results

Biochemical characterization of the mutant α spectrin. The affected family members (I.2, II.1, and III.1) had an additional band migrating slightly below the α-spectrin band when red cell membrane proteins were analyzed by SDS-PAGE (Fig. 2

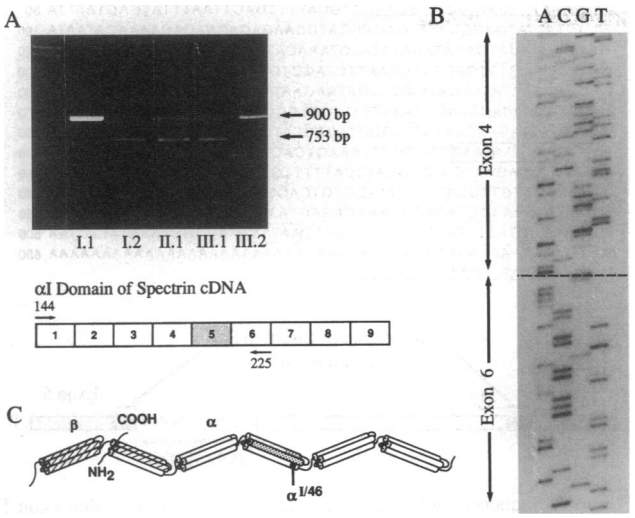

Figure 3. PCR amplification of reticulocyte α spectrin cDNA. (*A*) The sequence corresponding to the first 6 exons of α spectrin were amplified in the five family members using primers 144 and 225 shown in the diagram. The first lane is the molecular weight marker. Note the 753-bp fragment along with the expected 900-bp band in the affected members only (I.2, II.1, and III.1). Subcloning and sequencing of the 900-bp fragment in one patient revealed the normal sequence corresponding to part of the αI domain of α spectrin (data not shown). (*B*) The sequence corresponding to exon 5 was deleted from the 753-bp fragment as shown. This deletion of 147 bp did not alter the reading frame. (*C*) Schematic representation of the α spectrin–β spectrin heterodimer self-association site, showing the segment deleted from the α-spectrin protein (dotted area on α spectrin). This segment corresponds to helix 2 and part of helix 1 of the second triple helical unit of α spectrin, directly facing the $\alpha^{I/46}$ cleavage site located on helix 3.

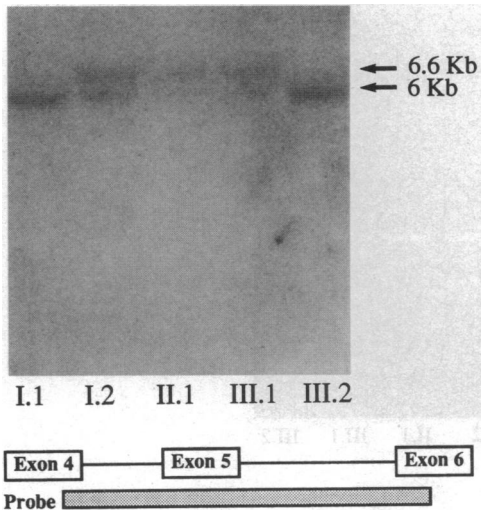

Figure 4. Southern analysis of genomic DNA. The probe used consisted of a genomic DNA fragment extending from exon 4 to exon 6 as shown. Genomic DNA samples from the five family members were digested with XbaI and analyzed by Southern blotting. Note the additional band of 6.6 kb in the affected family members only, suggesting the presence of an insertion within one of the two alleles.

A). Western blot analysis using polyclonal antibodies raised against α spectrin, β spectrin, and ankyrin revealed the α-spectrin origin of this extra band (data not shown). This truncated α spectrin protein had an apparent molecular weight of 235 kD and constituted only a small fraction (10%) of α spectrin on the membrane due to its instability and poor incorporation into the skeleton (26). Quantitation of the total amount of spectrin on the membrane by densitometric analysis of SDS-PAGE gels, and expressed as the ratio of spectrin to band 3, revealed a marked decrease in spectrin (spectrin to band 3 ratio of 0.66. Normal: 0.97±0.10) in patient II.1, the only family member with HPP. All other family members had a normal amount of spectrin on the membrane as assessed by this method (Fig. 1). Nondenaturing gel electrophoresis of spectrin extracts performed at 4°C showed a highly reproducible increase in the percentage of spectrin dimers out of the total pool of spectrin (i.e., spectrin dimers and spectrin tetramers), ranging from 19% (III.1); 25% (I.2) to 29% (II.1) (Normal being < 10%) (Fig. 2 *B*). Limited tryptic digestion of spectrin and separation of the fragments by SDS-PAGE (Fig. 2 *C*) or two-dimensional isoelectric-focusing/SDS-PAGE (Fig. 2 *D*), revealed an increase in the amount of a 46-kD peptide derived from the digestion of the αI domain, with a concomitant decrease in the amount of the normal 80-kD fragment. This peptide was transferred to a nylon membrane. After elution, direct amino acid sequencing localized the cleavage site at amino acid 258 (performed in the laboratory of Dr. P. Matsudaira at the Massachusetts Institute of Technology, Cambridge, MA). This cleavage site resides in helix 3 of the second triple helical repeat unit of α spectrin (21).

Elucidation of the molecular basis of the truncated α spectrin. PCR amplification of the cDNA sequence derived from the first 6 exons of the α spectrin gene revealed two fragments in all affected family members (I.2, II.1 and III.1) (Fig. 3 *A*), one of expected size (900 bp), and a shorter fragment (753 bp). Further analysis of the abnormal cDNA product revealed an in-frame deletion of the 147 bp sequence normally contributed by exon 5 (Fig. 3 *B*). The corresponding portion of the protein represents helix 2 and part of helix 1 of the second triple helical unit of α spectrin, directly opposite to the $\alpha^{I/46}$ cleavage site (21) (Fig. 3 *C*).

After excluding mutations of the splice junctions, genomic DNA was analyzed by Southern blotting using a genomic DNA probe extending from exon 4 to exon 6. In all affected family members, we found an additional band of higher molecular weight, suggesting the presence of an insertion within one of the two alleles in these patients (Fig. 4). This was verified by PCR amplification of a genomic DNA segment extending from exon 4 to exon 6. Using primers located within exon 4 (upstream primer 264) and exon 6 (downstream primer 268) (Fig. 5), a band of ~ 3.2 kb (expected size) was obtained in all five family members. An additional band of ~ 3.8 kb was also visualized in each affected member (I.2, II.1, and III.1), suggesting an insertion within one of the two alleles.

Subcloning and sequencing of this 3.8-kb fragment in one of the patients revealed a 632-bp insertion within exon 5 (Fig. 6). The overall primary structural organization of this insert is similar to that of mobile elements believed to transpose by means of RNA intermediates (1–4). Like these elements, this sequence is flanked by a target site duplication sequence (21 nucleotides) at the 5′ and 3′ ends which corresponds to nucleotides 780–800 of the α spectrin cDNA. It is terminated at the 3′ end by a poly–A tail (50 dAs), 21 nucleotides downstream from a consensus polyadenylation signal. Four other potential polyadenylation signals are present within the upstream se-

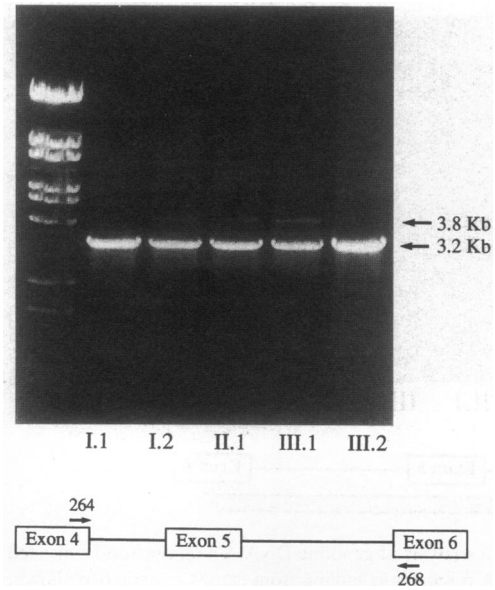

Figure 5. PCR amplification of α spectrin genomic DNA. Using primers located within exon 4 (upstream primer 264) and exon 6 (downstream primer 268), the PCR amplification of genomic DNA from all five family members, yielded a band of ~ 3.2 kb corresponding to the expected size. An additional band of ~ 3.8 kb was also visualized in the affected individuals only (I.2, II.1, and III.1), suggesting the presence of an insertion within one of the two alleles. This latter fragment was subcloned and sequenced in one patient.

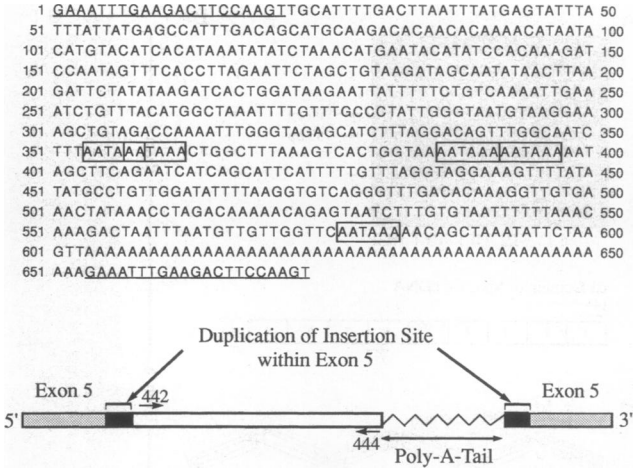

Figure 6. Sequence and schematic diagram of the insert within exon 5 of the α spectrin gene. This element was obtained by PCR amplification of genomic DNA of one of the patients as described in Fig. 5. Sequencing revealed 632 bp interrupting the normal sequence of exon 5. This insert is highly A-rich (overall 38% dA) and contains 5 consensus polyadenylation signals (*boxed*), two of them overlapping. The most 3′ signal is followed 21 bp downstream by a polyA tail constituted of 50 successive dA. No long open reading frame is present within the entire sequence as it is disrupted by multiple stop codons. The insert is flanked by a 21-bp sequence corresponding to nucleotides 780–800 of exon 5 at both 5′ and 3′ extremities (*underlined*), representing duplication of the insertion site. The sequence shown in this figure presents no homology to any previously reported sequence in both orientations. The two primers shown, 442 and 444, were used to amplify by PCR the insert without its poly–A tail. This fragment was used as probe for the Southern blot analysis shown in Fig. 7.

quence. The 3′ region is highly A-rich. This insert displays no long open reading frame, and may therefore be the 3′ untranslated region of a truncated mobile element. Alternatively, it may be a mobile element disrupted by mutations causing frameshifts or stop codons as seen in the majority of mobile elements. It has no homology to any previously reported sequence in both orientations.

Determination of the copy number of the insert by Southern blot hybridization. To search for the presence of other copies of this element, and to determine their number within the genome, DNA from 15 individuals belonging to five different nuclear families (i.e., child, father, and mother) was analyzed by Southern blot hybridization with the insert without its poly–A tail as probe. Using primers 442 (5′-AACTTAGAATATTTAGCT-3′) and 444 (5′-TGCATTTTGACTTAATTTAT-3′) (Fig. 6), the insert lacking its poly–A tail was amplified by PCR. This portion of the insert was labeled with ^{32}P and used as probe. Genomic DNA from the fifteen individuals was digested independently with two restriction enzymes which do not cut within the insert (HindIII and XbaI) and analyzed by Southern blot hybridization (Fig. 7). We were able to detect one to three bands in any given subject. The sizes of these bands varied among individuals; overall, four distinct bands of different mobilities were detected with the two restriction enzymes and none of the bands is common to all the individuals. This data along with the pattern of transmission within the families, is not consistent with a simple restriction enzyme polymorphism because it is confirmed using two different restriction enzymes. However, these findings are consistent with allelism of a specific gene, caused by its proximity to variable repetitive sequences responsible for variable band sizes. It is also possible, although less likely, that the polymorphic pattern seen on the Southern blot is due to the insertion of the mobile element in different locations within the genome. However, the pattern of transmission within the families studied could not prove this hypothesis.

Discussion

The DNA segment inserted within the α spectrin gene reported in this study shares characteristic features with the members of the human LINE-1 family (L1Hs), the only known family of human nonviral retrotransposons (for review see references 1–4). Although some of these highly repeated DNA elements appear to be functional and have large open reading frames, most are truncated at their 5′ end and disrupted by deletions, insertions, or polarity changes. Like these elements, the insert that we have characterized has no long open reading frame, has a long poly–A tail downstream from multiple consensus polyadenylation signals and its integration is accompanied by duplication of the insertion site which flanks the sequence. However, this insert has no homology to the previously reported members of the L1Hs family which, as a group, have a high degree of sequence homology (10–12). Likewise, this insert has no homology to the SINE elements and therefore does not belong to that family either. Alternatively, it could belong to a family of retropseudogenes. These DNA segments are usually nonfunctional elements derived from specific genes, but they lack intervening sequences and are dispersed at remote locations from that of the functional gene which is fixed (1–3). However, in this case, we would have expected one band on the Southern blots, common to all individuals, representing the locus of the

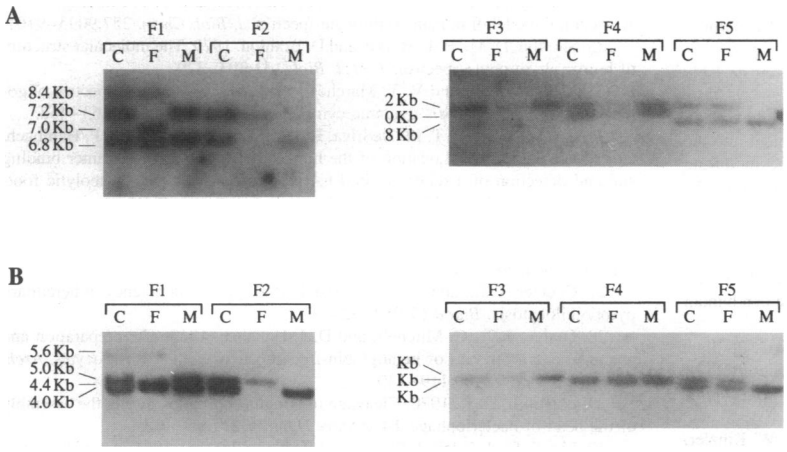

Figure 7. Determination of the copy number of the insert by Southern blot hybridization. Genomic DNA from fifteen individuals belonging to five (F1 to F5) unrelated nuclear families (i.e., Child [*C*], Mother [*M*], and Father [*F*]) was digested with two restriction enzymes independently and analyzed by Southern blot hybridization (*A*, digested with HindIII and *B*, with XbaI). The probe used consisted of the insert shown in Fig. 6 lacking its poly-A tail. One to three bands were detected in each individual. Overall, four distinct band sizes were seen and none of the bands is common to all the individuals. Family F1 represents three members belonging to the family reported in this paper (II.1, I.2, and I.1). Note that two of them (lanes *C* and *M*) have a unique band which has a molecular weight of 8.4 kb on the HindIII digest. This represents the element inserted within the α-spectrin gene and corresponds to the predicted size based on the known HindIII restriction map of the gene (44).

functional gene. It is conceivable that the absence of such a band reflects polymorphism due to variable repetitive sequences in the vicinity of the functional gene, causing minor variations in the band sizes among different alleles. The pattern of transmission within each family is consistent with this possibility. Alternatively, the variation of the bands on the Southern blots may represent variable locations of the mobile element within the genome. Regardless, the polymorphic pattern of hybridization seen in Fig. 7, whether due to variable repetitive sequences or to varying locations of this element among individuals, has not been, to our knowledge, described in other families of retropseudogenes. Moreover, this would represent the first evidence of active transposition within a family of retropseudogenes. Overall, the element described in this study, which is inserted in the α-spectrin gene does not conform to any of the previously described families of mobile elements and therefore, may represent a member of a new family.

The insertion of this mobile element within exon 5 of the α-spectrin gene resulted in skipping of this exon during splicing of the mRNA precursor. Exon skipping has previously been described in β spectrin (reviewed in 20) where point mutations in the 5′ donor consensus splice site resulted in excision of the upstream exon as well as shift in the reading frame. Several other cases of exon skipping have been reported in the literature, but they are almost all due to mutations altering the consensus splice sequences at the donor or acceptor splice sites flanking the involved exons (5, 39, 40). However, several studies have shown that manipulation of exons can affect splicing, and have suggested that alterations in the exon size or sequence could change the secondary structure of the RNA and thus influence the splice site selection (40, 41, 42). Other *Cis*- or *trans*-acting factors could potentially also play a role, although the exact molecular mechanism underlying the regulation of splicing are not yet fully understood. In the case of spectrin Dayton, the insertion of the mobile element has occurred at least 61 and 64 bp (excluding the 21 bp target site duplication) from the 5′ and 3′ ends of exon 5, respectively, but does not otherwise alter the sequence of this exon or that of the acceptor and donor splice sites. As suggested previously (5, 41, 42), this implies that factors other than consensus nucleotide sequences do contribute to the determination of the splice site selection.

The insertion of this mobile element within the α spectrin gene produces unique abnormalities in spectrin structure and function. The deletion of exon 5 does not alter the reading frame. Therefore, the resulting mutant protein is identical to the normal α-spectrin protein, except that it lacks the 49 amino acids contributed by exon 5, which have an approximate molecular weight of 5390 D. This is in agreement with the estimated size of 235 kD for the truncated α spectrin. The deletion of this portion of the protein eliminates helix 2 and part of helix 1 of the second triple helical repeat unit, exposing lysine 258 to tryptic cleavage, resulting in the abnormal 46-kD tryptic peptide. We have previously reported that the amount of RNA message of the mutant spectrin is normal but the protein is unstable, accounting for its under-expression on the membrane (26). Considering that this mutant spectrin constitutes only $\sim$ 10% of the total spectrin on the membrane, our finding of $\sim$ 20–30% of dimeric spectrin implies that this mutant spectrin is grossly dysfunctional. The location of the deletion within the second helix of the second triple helical unit further highlights the importance of this unit in the regulation of the spectrin dimer-dimer self association. It represents yet another unique defect which underlies the Sp α^{LV46} HE phenotype, in addition to previously reported mutations in helices 2 and 3 of this triple helical unit (for review see reference 43).

In conclusion, we have described a mobile element inserted within the gene of α spectrin, causing exon skipping and producing a unique truncated α-spectrin protein. This abnormal protein results in a clinical phenotype of hereditary elliptocytosis. Based on the primary structural organization of this element, its insertion within the α-spectrin gene, its unique sequence and the duplication of the insertion site, we conclude that it is a member of a novel family of mobile elements which transpose through RNA intermediates.

Acknowledgments

We are indebted to Diana Fraser for her help in typing this manuscript and to Donna Marie Mironchuk for preparing the photographic illustrations.

This work was supported by grants from The National Institutes of Health No K08 HL-02720 and HL-37462 and by grants from the South African Institute for Medical research and the Medical Research council of South Africa.

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