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Research Article

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Sequence and Exon–Intron Organization of the DNA Encoding the α I Domain of Human Spectrin

Application to the Study of Mutations Causing Hereditary Elliptocytosis

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Abstract

We have determined the exon–intron organization and the nucleotide sequence of the exons and their flanking intronic DNA in cloned genomic DNA that encodes the first 526 amino acids of the α I domain of the human red cell spectrin polypeptide chain. From the gene sequence we designed oligonucleotide primers to use in the polymerase chain reaction technique to amplify the appropriate exons in DNA from individuals with three variants of hereditary elliptocytosis characterized by the presence of abnormal α I spectrin peptides, 46–50 and 65–68 kD in size, in partial tryptic digests of spectrin. The α I/68-kD abnormality resulted from a duplication of leucine codon 148 in exon 4: TTG-CTG to TTG-TTG-CTG. The α I/50a defect was associated in different individuals with two separate single base changes in exon 6: CTG to CCG (leucine to proline) encoding residue 254, and TCC to CCC (serine to proline) encoding residue 255. In another individual with the α I/50a polypeptide defect, the nucleotide sequence encoding amino acid residues 221 through 264 was normal. The α I/50b abnormality resulted from a single base change of CAG (glutamine) to CCG (proline) encoding residue 465 in exon 11 in two unrelated individuals. In a third individual with α I/50b-kD hereditary elliptocytosis, the entire exon encoding residues 445 through 490 was normal. The relationship of the α I domain polypeptide structure to these mutations and the organization of the gene is discussed.

Introduction

The red cell membrane skeleton is composed of the proteins spectrin, actin, 4.1, and ankyrin, which interact to form an intricate lattice attached to the inner surface of the lipid bilayer (for reviews see references 1 and 2). This lattice provides the

mature red cell with structural support and flexibility during its lifespan. Defects in the various components of the membrane skeleton have been detected in hereditary elliptocytosis (HE),¹ hereditary pyropoikilocytosis (HPP), and hereditary spherocytosis (for reviews see references 1 and 3). HE is a heterogeneous disorder characterized by variable hemolytic anemia and elliptical red cell shape. Several abnormalities in the membrane skeleton have been identified in HE, including a number that have been localized to spectrin.

Spectrin, the major component of the membrane skeleton, is composed of dimers of α and β polypeptides, arranged in antiparallel fashion, that interact to form tetramers and larger oligomers (1, 2). Normal tetramer and oligomer assembly appear to be critical to the stability of the membrane skeleton as well as to normal red cell shape and function (1–3). The region of the heterodimer formed by interaction between the amino-terminal end of α spectrin and the carboxy-terminal end of β spectrin, referred to as the oligomer binding site, is of functional importance in tetramer and oligomer self-assembly (4).

Analysis of purified normal spectrin by limited digestion with trypsin and two-dimensional electrophoresis has allowed the identification of five α and four β spectrin peptide domains (5). The amino-terminal or α I domain of α spectrin is observed as an 80-kD fragment that is usually resistant to further digestion by trypsin in the mild conditions used (5). Using this method of analysis several abnormalities in the spectrin α I domain have been detected in individuals with HE. Recently the amino acid changes in several α I domain HE variants have been identified.

One HE variant, α I/68-kD HE (also referred to as α I/65 HE or $Sp\alpha^{1/65}$ HE), has been characterized by a 65–68-kD α I tryptic fragment (6–10). Amino acid analysis of spectrin from two unrelated individuals with this defect revealed a leucine insertion between residues 148 and 150 that results in an abnormal trypsin cleavage after arginine residue 131 (10). Two forms of HE have been characterized by the presence of separate α I tryptic fragments of 46–50 kD replacing the normal 80-kD α I tryptic fragment. One variant, α I/50a-kD HE (or $Sp\alpha^{1/46}$ HE; 11–13), has been found to be associated with two separate amino acid changes (10). In one individual a proline was substituted for lysine 254, resulting in trypsin cleavage after arginine 250; in a second individual serine 255 was replaced by proline, resulting in cleavage after lysine 252. A third form of elliptocytosis, α I/50b-kD HE, has been related to a substitution of proline for glutamine 465 in one affected individual, resulting in an aberrant cleavage by trypsin after argi-

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1. *Abbreviations used in this paper:* HE, hereditary elliptocytosis; HPP, hereditary pyropoikilocytosis; PCR, polymerase chain reaction.

nine 462 (10). In the same report, another $\alpha I/50b$ -kD HE variant was characterized by trypsin cleavage after arginine 464. In this individual, however, an amino acid change was not identified. Glutamine was present at position 465 and the following 28 residues were also found to be normal.

We have recently reported the isolation of a 17-kb α spectrin clone from a human genomic library (14). Approximately 3 kb of the 3' end of the genomic insert was sequenced and shown to contain three exons encoding amino acid residues 314 through 444 of the αI spectrin polypeptide (14). We have now mapped and sequenced the remaining exons in this large clone and two additional 3' exons in an overlapping clone. In all, 12 exons and their exon-intron boundaries have been sequenced, a region encoding the first 526 amino acid residues of the αI domain peptide. Using the α spectrin gene sequence information we designed three pairs of intronic oligonucleotide primers for use in the polymerase chain reaction (PCR) technique to amplify the appropriate exons in DNA from individuals with the $\alpha I/68$ -, $\alpha I/50a$ -, and $\alpha I/50b$ -kD variants of HE. The nucleotide sequences of the mutant alleles is presented.

Methods

Genomic cloning and exon mapping. The isolation of clone $\lambda 3021$ from a λ Charon 4a library of partially Eco RI-digested human DNA was previously described (14). The overlapping clone λ - α Sp-4a was isolated from a second library of human DNA partially digested with Hae III and Alu I (15) using a 1.7-kb Eco RI/Hind III fragment corresponding to the 5' end of subclone 3021 E1 (Fig. 1) as a screening probe. Identification of exons 8, 9, and 10 on the basis of nucleotide sequence has been previously described (14). The positions of the remaining exons were mapped by restriction enzyme digestion of the cloned genomic DNA, followed by Southern blotting (16) and hybridization to radiolabeled α spectrin cDNA probes. Most exons could be accurately mapped using restriction enzyme sites previously identified in α spectrin cDNA clones. The remaining exons were localized between infrequent restriction sites present within intronic regions. From 5' to 3' the 1.7-kb Eco RI-Hind III (exons 1 and 2), 2.2-kb Hind III-Xba I (exons 2 and 3), 4.7-kb Xba I-Xba I (exons 4-6), and the 1.3-kb Xba I-Hind III (exon 7) fragments of 3021 E1 were subcloned. The 1.6-(exon 11) and 0.75-kb (exon 12) Eco RI fragments of λ - α Sp-4a were also subcloned. Exon-containing restriction fragments were fur-

ther digested and subcloned into a double-stranded vector (pGEM; Promega Biotec, Madison, WI) for sequencing.

The α spectrin cDNA clones used in the exon mapping experiments were isolated from human bone marrow and fetal liver cDNA libraries. Clones were isolated by antibody screening of a λ gt11 cDNA expression library (17) with a polyclonal anti-spectrin antibody (18). Restriction fragments of these cDNAs and of the genomic α spectrin clone were used as screening probes to isolate additional 5' and 3' cDNA inserts.

Sequencing of subcloned genomic DNA and oligonucleotide synthesis. Subcloned genomic DNA fragments were sequenced using Klenow (Promega Biotec) or T7 DNA polymerase (Sequenase; United States Biochemical Corp., Cleveland, OH) and the dideoxy chain termination method of Sanger et al. (19). For some reactions synthetic oligonucleotides corresponding to cDNA or intronic regions were used as sequencing primers. Oligonucleotides were synthesized using an automated synthesizer (Applied Biosystems, Foster City, CA) and purified by gel electrophoresis or by column chromatography.

Genomic DNA amplification. The relevant α spectrin exons were amplified by the PCR technique (20, 21) using an automated DNA thermal cycler (Perkin-Elmer Cetus, Emeryville, CA). Briefly, 1 μ g of genomic DNA isolated from peripheral blood leukocytes was added to a 100- μ l reaction mixture consisting of 50 mM KCl, 10 mM Tris-Cl, pH 8.3, 1.5 mM MgCl₂, 0.01% (wt/vol) gelatin, 0.2 mM each of dATP, dCTP, dGTP, and dTTP, and 50 nmol of each oligonucleotide primer (Fig. 2). The reaction mixtures were first heated at 94°C for 5 min to inactivate any nucleases or proteases that might interfere with the reaction. After cooling, 2.5 U of Taq polymerase (Perkin-Elmer Cetus) were added to each sample. The reaction mixtures were then covered with mineral oil and amplified for 30 cycles. Each cycle consisted of 1.5 min at 94°C, 2 min at 55°C, and 3 min at 72°C. The last extension cycle at 72°C was lengthened to 10 min.

Subcloning of amplified DNA. PCR products were extracted once with phenol/chloroform, once with chloroform, and precipitated by ethanol at -20°C. The DNA was pelleted, washed once with 70% ethanol, dried, and dissolved in 50 μ l of water. The degree of amplification was determined by analyzing a portion of each PCR product by agarose gel electrophoresis and ethidium bromide staining. PCR products were blunt end ligated into Hinc II-digested pGEM4 plasmid DNA (Promega Biotec) using T4 DNA ligase (New England Biolabs, Beverly, MA) (22).

Direct sequencing of amplified DNA. Total genomic DNA was amplified as described above. Amplified DNA products were purified by agarose gel electrophoresis. The product band was excised and the DNA concentrated by electroelution and ethanol precipitation. Approximately one-tenth of the sample was then reamplified using a

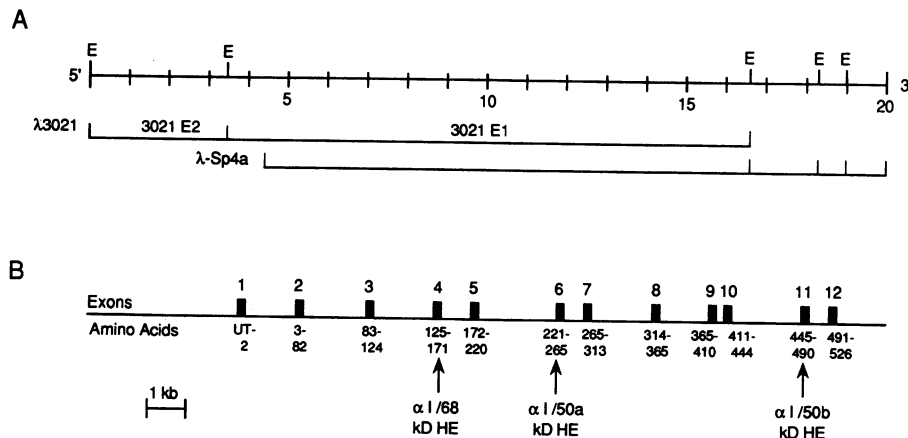


Figure 1. Exon organization of the gene region encoding the spectrin αI peptide domain. (A) Two overlapping clones $\lambda 3021$ (14), isolated from a library of human genomic DNA partially digested with Eco RI, and λ -Sp4a, isolated from a library made of human genomic DNA partially digested with Hae III and Alu I (15), are shown with a composite map of the Eco RI restriction sites (E) and genomic distances (in kilobases). (B) 12 exons were mapped by restriction enzyme digestion followed by Southern blotting and hybridization to ³²P-labeled cDNA fragment probes. Exons and adjacent intron regions were subcloned and sequenced (see Fig. 2). The amino acid residues of the αI peptide

sequence reported by Speicher and Marchesi (25) encoded by each exon are indicated. Exon 1 contains an untranslated region (UT) and coding sequence of the first two amino acids of the normal αI peptide. The locations of the $\alpha I/68$ -, $\alpha I/50a$ -, and $\alpha I/50b$ -kD HE amino acid substitutions (10) are shown.

rate-limiting amount (1 nmol) of one of the amplification primers and 50 nmol of the second primer (23). The amplified DNA was extracted once with phenol/chloroform and once with chloroform and passed through a spin column of Sephacryl S-300 (Pharmacia Fine Chemicals, Piscataway, NJ) in 10 mM Tris-Cl, pH 8.0, and 1 mM EDTA to remove excess primers. The eluate was concentrated by ethanol precipitation, washed with 70% ethanol, and dissolved in 50 μ l water. One-fifth of this sample was sequenced using T7 DNA polymerase and the rate-limiting PCR primer as the sequencing primer.

Results

Organization and nucleotide sequence of the 5' α -spectrin gene exons. The restriction map presented in Fig. 1 A was derived from two large overlapping genomic clones isolated from libraries of total human genomic DNA. Clone λ 3021, previously described by Linnenbach et al. (14), was isolated by hybridization to a synthetic 90-bp double-stranded DNA probe corresponding to a region of minimum codon degeneracy in the spectrin α I domain. Nucleotide sequence analysis of the 3' terminal 3 kb identified three exons, numbered 8, 9, and 10 in Fig. 1 B, which encoded amino acid residues 314–444 of the previously reported α spectrin polypeptide sequence (24, 25). Clone λ - α Sp-4a was isolated by hybridization to a fragment corresponding to the 5' end of the 13 kb Eco RI fragment (3021 E1) of clone λ 3021. To map the remaining exons in 3021 E1 we initially subcloned exon-containing fragments of intermediate size (see Methods) obtained by digestion with relatively infrequently cutting restriction enzymes. Exons were further localized within these subclones after digestion with more frequently cutting restriction enzymes, Southern blotting, and hybridization to 32 P-labeled α spectrin cDNA fragments. The shorter exon-containing fragments mapped in this manner were subcloned and sequenced.

As shown in Fig. 1 B, we have identified the first 12 coding exons of the human red cell α spectrin gene. The nucleotide sequences of all 12 exons and the immediately adjacent intron sequences are presented in Fig. 2. For completeness we have included the three exon sequences previously reported in reference 14. The exon DNA sequence is identical to the sequence separately determined for α spectrin cDNA encoding the same region (not shown). The translated amino acid sequence is in complete agreement with that previously determined by direct amino acid sequence analysis (24, 25). In all, the 12 exons encode the first 526 amino acid residues of the α I domain of spectrin. It should be noted that the amino acid sequence previously reported did not include the amino-terminal Met-Glu-Gln-Phe-Pro-Lys sequence that is predicted by the gene sequence. The use of limited trypsin digestion to generate the 80-kD α I peptide that was sequenced presumably resulted in the cleavage of the amino terminal 6 amino acids of the α spectrin chain after the lysine at position 6. The residue numbering in Figs. 1 and 2 conforms to the original published sequence.

The 12 exons varied in size from 43 to 240 bp. Intron size varied from 0.1 to 2.0 kb.

A compilation of the exon-intron junction sequences from Fig. 2 appears in Table I. The derived consensus 5' donor and 3' acceptor sequences of $\hat{C}AAG/gt_{gt}^{aa}$ and $(py)_{11}a_{aa}^{aa}ag/G$ are consistent with those reported for other intron-containing eucaryotic genes (26). All intron sequences immediately adjacent to exons obey the gt/ag rule (27). In all cases, there was no second

ag dinucleotide pair within the 15 bp upstream of the 3' splice junction. The 5' donor sequence of exon 5 contains a cryptic splice sequence within the intron immediately downstream from the correct splice signal. Use of this alternative splice sequence, however, would put the coding sequence out of frame as well as introduce several stop codons in the same reading frame immediately downstream. Except for exons 6 and 8, all 5' donor sequences end immediately after a triplet codon. Exons 6 and 8 end after the second base of the coding triplet, the third base of the codon being located at the start of the following exon. The same phenomenon occurs in a number of different eucaryotic genes.

Spectrin gene mutations in α I HE. Several defects in the α I domain of spectrin have been identified in individuals with HE (6–13). In α I/68-, α I/50a-, and α I/50b-kD HE, amino acid changes presumably responsible for the abnormalities have been reported (10). Determination of the exon organization and sequences encoding the α I domain of the protein has allowed us to undertake a study of the DNA sequence of the genes encoding these three HE variants.

In two unrelated individuals with the α I/68-kD HE defect, amino acid sequence analysis of the NH₂-terminal end of the abnormal 68-kD tryptic peptide revealed a leucine insertion between residues 148 and 150 (10). We amplified total genomic DNA from one of these individuals (H.P.) using the primers for exon 4 indicated in Fig. 2. The resulting 242-bp double-stranded product was cloned into the Hinc II site of pGEM4 and sequenced. Of the five subclones sequenced, three contained the normal sequence. Two subclones had a duplication of leucine codon 148: TTG-CTG to TTG-TTG-CTG. This result is shown in Fig. 3. Both strands of one mutant subclone were sequenced and found to agree completely with the entire normal exon sequence except for the TTG leucine codon duplication.

The α I/50a-kD HE abnormality has been shown to be associated with separate proline substitutions involving either serine 255 or leucine 254 (10). These changes map to exon 6 (Figs. 1 B and 2). Using oligonucleotide primers P16 and P6 indicated in Fig. 2 we amplified total genomic DNA from one of these individuals (T.S.). DNA sequence analysis of the subcloned normal and mutant exons is shown in Fig. 4 A. A single base change of CTG to CCG in exon 6 in one allele causes a substitution of proline for leucine 254.

The mutation of another individual (A.R.) with the α I/50a peptide abnormality was also studied. Using oligonucleotide primers P5 and P6, we amplified total genomic DNA. The PCR product was gel purified and reamplified using a rate-limiting amount of the 3' primer (oligonucleotide P6). The total asymmetrically amplified DNA product was then directly sequenced using primer P6 and T7 DNA polymerase (Sequenase). This procedure results in the simultaneous sequencing of the amplified exon 6 from both normal and mutant alleles. As shown in Fig. 4 B, this procedure detected a single base change of TCC to CCC in codon 255 of one allele resulting in the substitution of proline for serine. This initial analysis used a 5' primer (P5) located within the exon sequence, and, as a result, did not allow a sequence analysis of the complete exon. We subsequently confirmed the presence of a single base change in the exon of the mutant allele by using primers P16 and P6, and sequencing after subcloning (not shown).

Genomic DNA from another individual (M.A. in reference 11) with HPP and an associated α I/50a-kD defect was also

Exon 1

tgaattcgaactggacagttccatttgaattattttctctctctctctctctctctctgacacattttatcttgcagGTTCTAAAC
 CCTTAGGAAAAATGGAGCAATTTCCAAAGGAAACCgtgagtacatattttctctctcatgcaatttgctcattaatattattagg
 MetGluGlnPheProLysGluThr
 1

Exon 2

agaactagcaattaacagaatctttttaacttccagGTTGTGGAGAGCAGTGGCCAAAGGTTTTGAAACAGCAGAAGAGATC
 ValValGluSerSerGlyProLysValLeuGluThrAlaGluGluIle
 3 10
 CAGGAGAGCGCTCAGGAAGTGTGACTCGGTATCAAAGTTTCAAAGGAGCGGTTGCTGAGAGGGTCAAGCCTTGAGGATTCC
 GlnGluArgArgGlnGluValLeuThrArgTyrGlnSerPheLysGluArgValAlaGluArgGlyGlnLysLeuGluAspSer
 20 30 40
 TATCACTTACAAGTTTCAAGCGAGATGCAGATGATCTGGGGAAGTGGATCATGGAGAACTCAATATCTTAACCGATAAGAGC
 TyrHisLeuGlnValPheLysArgAspAlaAspAspLeuGlyLysTrpIleMetGluLysValAsnIleLeuThrAspLysSer
 50 60 70
 TATGAAGACCCAACTAATATACAGgtcactcagttctcttctgattgctcagagcagaccctaaga
 TyrGluAspProThrAsnIleGln
 80

Exon 3

gaagactgtgcttcattagacaaaaactctgatttctaaacttttagGGGAAATATCAGAAGCATCAATCCCTTGAAGCAGAGGT
 GlyLysTyrGlnLysHisGlnSerLeuGluAlaGluVa
 83 90
 GCAAACGAAATCAAGACTCATGTCTGAACTGGAAAAACAAGGGAAGAACGATTTACCATGGGTCATTCTGCCACGAAAGAAAC
 lGlnThrLysSerArgLeuMetSerGluLeuGluLysThrArgGluGluArgPheThrMetGlyHisSerAlaHisGluGluTh
 100 110 120
 GAAGgtatatgtatgggccccttgaagaagttccaattattgattatggtatcagggttttaactgatt
 rLys
 124

Exon 4 (α I/68 kD HE)

oligo P1
tcctgctcccagtgctgtggtgagtggttttgccectctttcctaagGCCCATATAGAGGAGCTACGCCACCTGTGGGAC
 AlaHisIleGluGluLeuArgHisLeuTrpAsp
 125 130 #
 CTGCTGTTAGAGCTGACCTGGAGAAGGCTGACCACTGCTGCGGGCCCTGAAGTTCCAGCAGTATGTACAGGAGTGTGCTGAC
 LeuLeuLeuGluLeuThrLeuGluLysGlyAspGlnLeuLeuArgAlaLeuLysPheGlnGlnTyrValGlnGluCysAlaAsp
 140 Δ 150 160
 Leu
 α I/68
 kD HE
 ATCTTAGAGTGGATTGGAGACAAAGgtatcgacttaaacagcaatcacacagggcctcccactcctctgtccc
 IleLeuGluTrpIleGlyAspLys oligo P4
 171

Exon 5

caactcaatcaactctctgtgtctacagGAGGCTATAGGGACATCAGTGGAGCTAGGTGAAGACTGGAGCGCACCGAAGTTCTG
 GluAlaIleAlaThrSerValGluLeuGlyGluAspTrpGluArgThrGluValLeu
 172 180 190
 CATAAGAAATTTGAAGACTTCCAAGTGGAGCTGGTAGCTAAAGAAGGGAGAGTTGTTGAAGTGAACCAATATGCCAATGAGTGT
 HisLysLysPheGluAspPheGlnValGluLeuValAlaLysGluGlyArgValValGluValAsnGlnTyrAlaAsnGluCys
 200 210
 GCCGAGgtgaggtgggagcaaatattactccgtgcaaagggttcttcttatacttaagaata
 AlaGlu
 220

Figure 2. Nucleotide sequence of α spectrin gene exons and adjacent intron regions. Exon sequences are indicated in capital letters, intron sequences in lowercase letters. The numbering of the amino acid residues conforms to that reported for the α I spectrin peptide (24, 25). The locations of the leucine insertion (Δ) and the abnormal trypsin cleavage site (*) in α I/68-kD HE (exon 4), and the proline substitutions (\dagger) and abnormal trypsin cleavage sites (*) in α I/50a-(exon 6) and α I/50b-(exon 11) kD HE are shown. The nucleotide sequences of exons 8, 9, and 10 were previously reported (14). The DNA sequences used as oligonucleotide primers in PCR experiments are underlined.

Exon 6 (α I/50a kD HE)

oligo P16
ctcatctctgtataaetccaggtgtgttttccttcaacagGAAAACCATCCTGACCTACCCTTAATTCAGTCTAAGCAAAATGA
 GluAsnHisProAspLeuProLeuIleGlnSerLysGlnAsnG1
 221 230

oligo P5
 GGTGAATGCTGCCTGGGAGCGCCTTCGTGGTTGGCTCTCCAGAGACAGAAAGCTCTGTCCAATGCTGCAAACTTACAACGATT
 uValAsnAlaAlaTrpGluArgLeuArgGlyLeuAlaLeuGlnArgGlnLysAlaLeuSerAsnAlaAlaAsnLeuGlnArgPh
 240 250# # † † 260

ProPro
 α I/50a
 kD HE

CAAAAgtatggatctggccactgctttatagaaaactttgaagtgctttataaaaaccgtgctttgtattaggctct
 eLysAr
 265 oligo P6

Exon 7

atcttctttggttgagactctgttaatgtctgcagGGATGTGACTGAAGCCATCCAGTGGATCAAGGAGAAGGAACCTGTACT
 gAspValThrGluAlaIleGlnTrpIleLysGluLysGluProValLe
 266 270 280

CACCTCTGAGGACTATGGCAAAGACCTTCTTGCCTCTGAAGGACTGTTTCACAGCCACAAGGGACTTGAGAGAAATCTTGCAGT
 uThrSerGluAspTyrGlyLysAspLeuValAlaSerGluGlyLeuPheHisSerHisLysGlyLeuGluArgAsnLeuAlaVa
 290 300

CATGAGTGACAAGgtaatgcgctgtagagaaatggctaaccggttgagctagaa
 lMetSerAspLys
 310

Exon 8

agagtacttgatcttctactaggtttcttttccatcccaaagGTGAAGGAGTTATGTGCTAAAGCAGAGAAGCTGACACTTTC
 ValLysGluLeuCysAlaLysAlaGluLysLeuThrLeuSe
 314 320

CCATCCTTCAGATGCACCTCAGATCCAGGAGATGAAAGAAGATCTGGTCTCCAGCTGGGAGCATATTCGTGCCCTGGCCACCAG
 rHisProSerAspAlaProGlnIleGlnGluMetLysGluAspLeuValSerSerTrpGluHisIleArgAlaLeuAlaThrSe
 330 340 350

CAGATATGAAAACTGCAGGCTACTTATTGgtgggaaatccctcccctttattgctctacctatct
 rArgTyrGluLysLeuGlnAlaThrTryTr
 360 365

Exon 9

tttctccttctgtcttttacctcacttttcccacagGTACCATCGATTTTCATCTGACTTTGATGAACTCTCAGGCTGGATGA
 pTyrHisArgPheSerSerAspPheAspGluLeuSerGlyTrpMetA
 366 370 380

ACGAGAAGACTGCTGCGATCAATGCTGATGAGCTGCCAACAGATGTGGCTGGTGGAGAAGTTCTGCTGGACAGGCATCAGCAGC
 snGluLysThrAlaAlaIleAsnAlaAspGluLeuProThrAspValAlaGlyGlyGluValLeuLeuAspArgHisGlnGlnH
 390 400

ATAAGgtagagaagaaaggctgcccagtaggaggaggggcgag
 isLys
 410

Exon 10

gcattttgagacactttgtttgtggccacagCATGAGATTGACTCTTACGATGACCGATTTCAATCTGCTGATGAGACTGGTC
 HisGluIleAspSerTyrAspAspArgPheGlnSerAlaAspGluThrGlyG
 411 420

AAGACCTCGTGAATGCCAATCATGAAGCCTCTGATGAAGTTCGGGAAAAGgtaatctagtttaacagagtttgt
 lnAspLeuValAsnAlaAsnHisGluAlaSerAspGluValArgGluLys
 430 440 444

Figure 2 (Continued)

Exon 11 (α I/50b kD HE)

oligo P19
cttccatatacattatctcctctcttcccaagATGGAAATACTTGACAACAAGTGGACTGCCCTGCTGGAAGTGTGGGACGAGC
MetGluIleLeuAspAsnAsnTrpThrAlaLeuLeuGluLeuTrpAspGluA
445 450 460

GTCATCGTCAGTATGAGCAGTGCCTGGACTTTCATCTCTTCTACAGAGACAGTGGCAAGTGGACAGTTGGATGAGTAGACAAG
rgHisArgGlnTryGluGlnCysLeuAspPheHisLeuPheTyrArgAspSerGluGlnValAspSerTrpMetSerArgGlnG
† 470 480 4

Pro
 α I/50b
kD HE

AGgtaacgggagggggtccataccatctctagaagtaattttctctcacccttcatttgccaccatgactaccatgagttccctca
lu
90 oligo P9

Exon 12

acagcttaggtgtgtctcttctgtctctctacagGCCTTCTGGAAAACGAGGATCTGGGAAACTCACTGGGCAGTGCAGAAGCCC
AlaPheLeuGluAsnGluAspLeuGlyAsnSerLeuGlySerAlaGluAlaL
491 500

TTCTTCAGAAGCATGAAGACTTTGAGGAAGCCTTTACTGCCAGGAAGAGAAGATCATAgtaagaaattggccctagtttgggc
euLeuGlnLysHisGluAspPheGluGluAlaPheThrAlaGlnGluGluLysIleIle
510 520 527

attggetccctctctgtatacataa

Figure 2 (Continued)

studied. Although the precise site of abnormal cleavage by trypsin in α spectrin from MA has not been identified, the abnormal α I/50-kD tryptic peptide produced appeared to be identical in mobility, after two-dimensional electrophoresis, to that derived from α spectrin of T.S. for which actual amino acid sequence was obtained. Genomic DNA from MA was amplified using oligonucleotide primers P5 and P6 and directly sequenced as described above. In addition, 18 individual subclones of the PCR product using oligonucleotide primers P16 and P6 were sequenced. No abnormalities were detected in the DNA sequence of exon 6 which encodes residues 221 through 264 (i.e., 29 residues upstream and 14 residues downstream from the abnormal trypsin cleavage site identified in T.S.).

A third abnormality of the α I domain has been described in individuals with HE (10). This variant, referred to as α I/50b-kD HE, is characterized by a 50-kD α I tryptic peptide and two smaller fragments of 17 and 19 kD not observed in the α I/50a-kD HE defect. Sequence analysis of the amino-terminal ends of the 17- and 19-kD tryptic fragments from one individual (H.B.) revealed a substitution of proline for glutamine at position 465 (10). This change maps to α spectrin exon 11 (Figs. 1 B and 2). We amplified total genomic DNA from HB and a second unrelated affected individual using the primers indicated. The DNA products were subcloned into pGEM and sequenced (Fig. 5). In both individuals we detected a single base change of CAG (glutamine) to CCG (proline) in codon 465 of exon 11 in one allele.

Spectrin from a third α I/50b-kD HE individual was previously studied (D.F. in reference 10). An amino acid change responsible for the abnormal tryptic peptides was not identified although limited tryptic digestion of α spectrin produced peptides similar to those of HB. Sequence analysis of the amino-terminal ends of the 19 and 17 kD peptides identified the cleavage site as arginine 464, instead of arginine 462 as in

H.B., but revealed no amino acid changes in the following 28 residues. Genomic DNA from DF was amplified and subcloned, and the entire exon 11 was sequenced as described above. A total of 20 separate subclones were sequenced. A single subclone had an AAC (asparagine) to GAC (aspartate) change at position 450. In 19 additional subclones sequenced, however, this base change was not detected and the DNA sequences were completely normal. The base change noted in the single subclone was most likely due to an error introduced during amplification by the Taq polymerase. We have concluded that the entire exon 11, encoding 19 amino acid residues upstream and 26 amino acid residues downstream from the abnormal trypsin cleavage site after arginine 464, is normal in both alleles of D.F.

Discussion

Molecular cloning of the gene region encoding the NH₂ terminus of human erythroid α spectrin is of interest for several

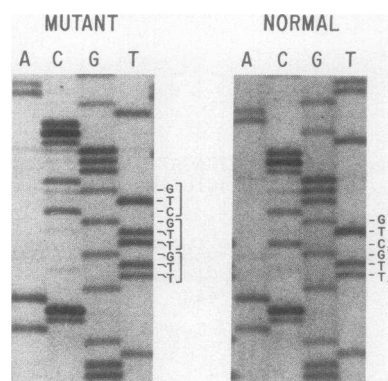


Figure 3. Nucleotide sequence of the α I/68-kD HE gene mutation. Genomic DNA from peripheral blood leukocytes of heterozygous patient H.P. was amplified using Taq polymerase and the oligonucleotide primers for exon 4 indicated in Fig. 2. The product DNA was subcloned and sequenced. The relevant regions of exon 4 of the normal allele (TTG-CTG) and the mutant allele (TTG-TTG-CTG) containing the duplicated leucine codon 148 are shown.

Table I. Nucleotide Sequences of α Spectrin Exon Donor/Acceptor Splice Sites

Intron	5' Donor sequence (exon/intron)	3' Acceptor sequence (intron/exon)
1	CCAAAGGAAACC/gtgagtacatatttc ProLysGluThr	cttttaactccag/GTTGTGGAGAGC ValValGluSer
2	ACTAATATACAG/gtcactcagtctct ThrAsnIleGln	atttataaacttag/GGAAAATATCAG GlyLysTyrGln
3	GAAGAAACGAAG/gtatatgtatgggcc GluGluThrLys	ccctcttctctaaag/GCCCATATAGAG AlaHisIleGlu
4	ATTGGAGACAAG/gtatcgaacttaaaa IleGlyAspLys	cttctgtgtctacag/GAGGCTATAGCG GluAlaIleAla
5	GAGTGTGCCGAG/gtgaggtgggagcaa GluCysAlaGlu	gttttcttcaacag/GAAAACCATCCT GluAsnHisPro
6	ACGATTCAAAAAG/gtatgatctggcca nArgPheLysAr	tgtaatgtctgcag/GGATGTGACTGA gAspValThrGl
7	ATGAGTGACAAG/gtaatcgctgtag MetSerAspLys	ttttcatcccaaag/GTGAAGGAGTTA ValLysGluLeu
8	GGTACTTATTG/gtgggaaatccctcc nAlaThrTryTr	tcacttttcccacag/GTACCATCGATT pTyrHisArgPh
9	CAGCAGCATAAG/gtagagaagaagca GlnGlnHisLys	gttttggccacag/CATGAGATTGAC HisGluIleAsp
10	GTTTCGGAAAAG/gtaactctagttaac ValArgGluLys	tccttcttccaaag/ATGGAAATACTG MetGluIleLeu
11	AGTAGACAAGAG/gtaacggaggggctc SerArgGlnGlu	ttctgtcttctacag/GCCTTCCTGGAA AlaPheLeuGlu
12	GAGAAGATCATA/gtaagaattggccc GluLysIlelle	

Consensus sequences:

α spectrin, $\hat{C}AAG/gt_{ga}^{\wedge}$ (py)₁₁a₂ag/G

other eucaryotic genes (reference 26), $\hat{C}AG/gt_{ga}^{\wedge}agt$ (py)₁₁n₁ag/G

reasons. First, a number of defects involving the NH₂-terminal or α I domain of spectrin have been detected in HE and HPP. Knowledge of the exon sequences and exon-intron organization of the region encoding the α I domain of spectrin will facilitate the study of these mutations. Second, an understanding of the organization of the erythroid α spectrin gene may provide significant insight into the molecular evolution of the spectrin gene and other members of the gene family to which it belongs. Finally, although not the subject of this report, the identification of the α spectrin promoter would allow studies on the mechanism of tissue-specific expression of the α spectrin and other genes expressed exclusively during erythroid cell differentiation.

We have reported here the DNA sequences and the organization of the first 12 coding exons of the human erythroid α spectrin gene. The coding region studied spans ~ 17 kb and encodes the first 526 amino acid residues of the α I domain. Given a total estimated length of ~2,400 amino acids for α spectrin, the entire gene is predicted to be composed of ~ 55 exons and to be ~ 80 kb in size, assuming a similar frequency and length of introns in the remainder of the gene.

The α spectrin polypeptide has been previously shown to be a long rod-shaped molecule composed of ~ 20 homologous

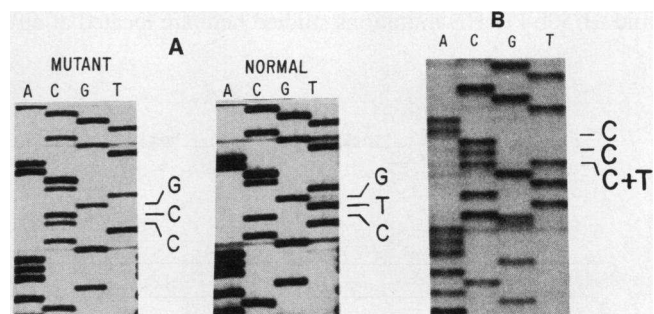


Figure 4. Nucleotide sequence of two separate α I/50a-kD HE gene mutations. (A) Genomic DNA from T.S., heterozygous for an α I/50a-kD protein defect, was amplified using Taq polymerase and the oligonucleotide primers P16 and P6 for exon 6. The product DNA was subcloned and sequenced. The CTG encoding leucine 254 in the normal allele is changed to CCG encoding proline in the mutant allele. (B) Genomic DNA from a second heterozygous individual (A.R.) was amplified using oligonucleotide primers P5 and P6. The product DNA was gel purified, reamplified asymmetrically using a rate-limiting amount of primer P6, and sequenced directly. The sequence reveals the TCC serine codon 255 of the normal allele and a single base change to CCC encoding proline in the exon of the mutant allele.

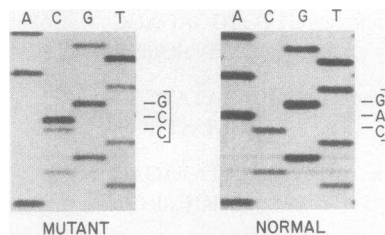


Figure 5. Nucleotide sequence of the α 1/50b-kD HE mutation. Genomic DNA from a heterozygous individual (H.B.) was amplified using Taq polymerase and the oligonucleotide primers for exon 11 indicated in Fig. 2. The DNA product was subcloned and sequenced.

106 amino acid repeats, each of which can be folded into a triple helical structure connected to adjacent repeats by a short nonhelical connecting region (25). The high degree of homology between the individual repeat units, as well as the conservation of a triple helical conformation in each repeat segment, suggest that the gene evolved by repeated duplication events of a smaller DNA domain. In the region of the gene that we have studied, however, there is no obvious relationship between the exon-intron organization of the gene and the repeat structure of the polypeptide. Exon size varied from 43 to 240 bp, while intron size varied from 0.1 to 2.0 kb. As shown in Fig. 6, each repeat unit of the α 1 domain is interrupted by one or more exon-intron junctions. Furthermore, no single exon or grouping of adjacent exons encodes a distinct 106 amino acid repeat unit and none of the splice junctions defines the end of a repeat. This is different from the situation in the gene encoding dystrophin, the protein that is defective in Duchenne muscular dystrophy and a member of the extended family of spectrinlike proteins. In dystrophin, which is composed largely of repeat units of 109 amino acid residues similar to those in spectrin, the ends of the first six protein repeats appear to be defined exactly by exon-intron boundaries (28). A more complete understanding of the molecular evolution of the α spectrin gene must await exon-intron mapping of the entire gene.

It is apparent in Fig. 6, however, that the α 1/68-, α 1/50a-, and α 1/50b-kD HE mutations studied here are located at ap-

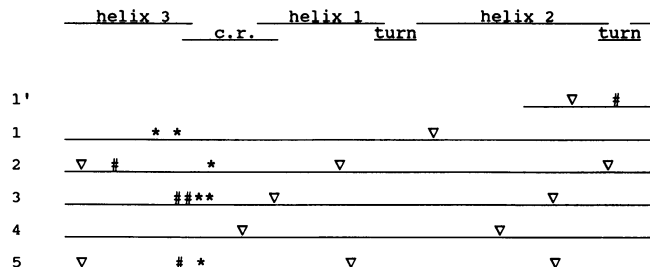


Figure 6. Relationship of α spectrin polypeptide repeat units to exon-intron organization and HE mutations. The first five 106 amino acid repeat units (1-5) and the amino-terminal end (1') previously described (25) are shown with the approximate locations of the exon-intron junctions (∇), amino acid changes (*), and abnormal trypsin cleavage sites (#) in α 1/68-(repeat 2), α 1/50a-(repeat 3), and α 1/50b-(repeat 5) kD HE. The amino acid changes and the abnormal trypsin cleavage site in α 1/78-kD HE (29, 30) are also indicated (in 1 and 1'). The approximate regions in each repeat segment comprising helix 1, 2, and 3, and the connecting region (c.r.) are also indicated.

proximately the same position (residues 25, 26, or 27) in the 106 amino acid lengths of repeats 2, 3, and 5 of α spectrin. Each of these mutations occurs at the junction between the COOH-terminal end of helix 3 and the region postulated to connect adjacent triple helical structures referred to as the connecting region in the model of spectrin folding proposed by Speicher and Marchesi (25). These amino acid changes may exert their effect by altering critical relationships within the α spectrin polypeptide or by disturbing interchain relationships between the antiparallel α and β spectrin polypeptides. The end result is probably a functionally significant change in conformation of the important head-end or oligomer binding site of the spectrin heterodimer resulting in impaired spectrin dimer self-association. Two additional amino acid substitutions have recently been described in HE variants associated with an α 1/78-kD tryptic fragment (29, 30). Interestingly, the protein defects in these cases are located in approximately the same position (residues 18 and 22) of repeat 1 of α spectrin.

The present experiments confirm the nature of the previously described amino acid changes associated with α 1/50a- and α 1/50b-kD HE by demonstrating the specific single base substitutions in the α spectrin gene of affected individuals. Both of these anomalies are heterogeneous at the molecular level. Two separate nucleotide changes resulting in two different amino acid replacements were observed in α 1/50a-kD HE. A third α 1/50a defect in M.A., not yet specifically characterized, is different from these two. At least two different defects are associated with α 1/50b-kD HE: a single nucleotide change resulting in a proline substitution for glutamine 465 detected in two unrelated individuals, and an as yet unidentified but different mutation in individual D.F.

In contrast, α 1/68-kD HE appears to be the result of a single gene mutation. The same TTG codon duplication has been observed in nine individuals from five separate families from North America and France (31) and in five unrelated individuals from North Africa (32). The occurrence of the same mutation in all individuals studied is unlikely to be the result of multiple separate identical mutation events. Instead, it probably reflects a founder effect. The relatively high incidence of α 1/68-kD HE in Central West Africa (33) suggests that the original mutation spread from this region to Northern Africa and, subsequently, to North America and Europe. It is tempting to speculate that the relatively high frequency of this HE defect in black individuals reflects some type of beneficial effect in the heterozygous state, similar to the situation in the sickle cell trait where individuals are protected against malaria infections. As yet, however, no direct evidence for this possibility exists.

The duplication of leucine codon 148 in α 1/68-kD HE is probably the result of the process called frameshift mutagenesis (34), in which there is an intrachromosomal mispairing of sister chromatids during meiosis in regions of short directly repeated sequences. Such direct repeats, as small as two nucleotides, have been shown to be associated with both deletions and insertions in the human globin genes (35-37).

Most known amino acid changes causing the various types of HE occur close to the site of abnormal tryptic cleavage of the α 1 domain. The mutations thus far identified that are located the furthest away from the abnormal tryptic cleavage site occur in α 1/78-kD HE where the amino acid replacements are at residue 35 or 39 and the abnormal cleavage at residue 10; i.e., 25 and 29 residues proximal to the mutation (29, 30). In

two individuals studied, one with $\alpha I/50a$ -kD HE and one with $\alpha I/50b$ -kD HE, a gene mutation was not identified in the exon encoding the site of the abnormal tryptic cleavage. In the case of the $\alpha I/50a$ variant this exon encodes 29 and 14 amino acid residues to either side of the cleavage site. In the $\alpha I/50b$ variant the exon encodes 19 and 28 amino acid residues to either side of the cleavage site. Thus, the protein defects in these two individuals may be located more distally (or proximally) to the abnormal tryptic cleavage site in neighboring exons of the α spectrin gene. Or, as recently suggested in the case of one $\alpha I/74$ -kD HE variant (38), the aberrant αI tryptic cleavage may not be the result of a mutation in the α spectrin chain, but, rather, a mutation in the apposing β spectrin chain.

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