

## Low expression allele alpha LELY of red cell spectrin is associated with mutations in exon 40 (alpha V/41 polymorphism) and intron 45 and with partial skipping of exon 46.

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### Research Article

The alpha V/41 polymorphism of erythroid alpha-spectrin has been characterized initially by an increased susceptibility to proteolysis of the alpha IV-alpha V domain junction (Alloisio N., L. Morlé, J. Maréchal, A.-F. Roux, M.-T. Ducluzeau, D. Guetarni, B. Pothier, F. Baklouti, A. Ghanem, R. Kastally, et al. 1991. *J. Clin. Invest.* 87:2169-2177). Until now, it has been found associated invariably with a low expression level of the corresponding alpha chain. Among 61 chromosomes investigated in French and North African individuals or kindreds, we observed 19 chromosomes with the alpha V/41 polymorphism. With no single exception, the latter displayed a point mutation in exon 40 (Leu-->Val; CTA-->GTA) at position alpha 1857. According to the triple helical model of spectrin structure, this change accounts for the peptide maps' abnormalities. Sequencing the entire alpha V domain cDNA disclosed, in addition, a partial skipping of exon 46. At the gene level, a substitution (C-->T) was evidenced at nucleotide -12 of intron 45. This mutation appeared linked to the exon 40 mutation in 17 chromosomes, again with no single exception, among 53 examined chromosomes. We hypothesized that the lack of exon 46 would hamper the nucleation process and eventually account for the low expression feature. The present doubly mutated allele was renamed allele alpha LELY (low expression, Lyon).

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# Low Expression Allele $\alpha^{\text{LELY}}$ of Red Cell Spectrin Is Associated with Mutations in Exon 40 ( $\alpha^{\text{V}/41}$ Polymorphism) and Intron 45 and with Partial Skipping of Exon 46

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## Abstract

The  $\alpha^{\text{V}/41}$  polymorphism of erythroid  $\alpha$ -spectrin has been characterized initially by an increased susceptibility to proteolysis of the  $\alpha\text{IV}-\alpha\text{V}$  domain junction (Alloisio N., L. Morlé, J. Maréchal, A.-F. Roux, M.-T. Ducluzeau, D. Guetarni, B. Pothier, F. Baklouti, A. Ghanem, R. Kastally, et al. 1991. *J. Clin. Invest.* 87:2169–2177). Until now, it has been found associated invariably with a low expression level of the corresponding  $\alpha$  chain. Among 61 chromosomes investigated in French and North African individuals or kindreds, we observed 19 chromosomes with the  $\alpha^{\text{V}/41}$  polymorphism. With no single exception, the latter displayed a point mutation in exon 40 (Leu  $\rightarrow$  Val; CTA  $\rightarrow$  GTA) at position  $\alpha$  1857. According to the triple helical model of spectrin structure, this change accounts for the peptide maps' abnormalities. Sequencing the entire  $\alpha\text{V}$  domain cDNA disclosed, in addition, a partial skipping of exon 46. At the gene level, a substitution (C  $\rightarrow$  T) was evidenced at nucleotide -12 of intron 45. This mutation appeared linked to the exon 40 mutation in 17 chromosomes, again with no single exception, among 53 examined chromosomes. We hypothesized that the lack of exon 46 would hamper the nucleation process and eventually account for the low expression feature. The present doubly mutated allele was renamed allele  $\alpha^{\text{LELY}}$  (low expression, Lyon). (*J. Clin. Invest.* 1993. 91:2091–2096.) **Key words:** red cell •  $\alpha$ -spectrin • hemolytic anemia • point mutations • gene expression

## Introduction

The red cell skeleton is a protein assembly that laminates the inner surface of the erythrocyte membrane (for review see references 1–4). Spectrin, an  $\alpha\beta$  dimer, represents a major protein of the skeleton. Using partial tryptic digestion, the  $\alpha$  chain was dissected into five domains ( $\alpha\text{I}$  to  $\alpha\text{V}$ ,  $\text{NH}_2$  to  $\text{COOH}$  termini) (5). The  $\alpha$  chain contains 2,429 amino acids (6, 7). Spectrin  $\alpha$  gene has 52 exons (8). In avian (for review see reference 9) and mammalian species (10), spectrin  $\alpha$  chain is synthesized in large excess, a point that will prove to be important in the present work.

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It has long been noted that hereditary elliptocytosis (HE)<sup>1</sup> associated with  $\alpha$  chain mutations has a variable expression, even within a given family. This fact has suggested (11, 12) that some low expression (LE)  $\alpha$  alleles, occurring in *trans* to a  $\alpha^{\text{HE}}$  allele, could account for the enhanced expression of the  $\alpha^{\text{HE}}$  allele. A preliminary study on the basis of haplotype determination supported this assumption (13). Alloisio et al. (14) then characterized a protein phenotype associated with the LE character. Spectrin peptide maps disclosed an augmented susceptibility to proteolysis of the  $\alpha\text{IV}-\alpha\text{V}$  domain junction. Despite the lowered proportion of the altered  $\alpha$  chain, the final outcome was a significant increase of the  $\alpha\text{V}$  41-kD fragment. The corresponding allele was initially designated  $\alpha^{\text{V}/41}$  allele (14). Indirect data led to the estimate that membrane incorporation of the  $\alpha^{\text{V}/41}$  chain was reduced by  $\sim 50\%$ , as has been detailed (14, 15). The  $\alpha^{\text{V}/41}$  polymorphism remained asymptomatic in the heterozygous or in the homozygous state presumably because of the fact that enough  $\alpha^{\text{V}/41}\beta$  dimers are still synthesized. Although the  $\alpha/\alpha^{\text{HE}}$  diplotype yielded asymptomatic to mild HE, the  $\alpha^{\text{V}/41}/\alpha^{\text{HE}}$  diplotype produced a moderate to severe picture, achieving hereditary pyropoikilocytosis in a number of cases, depending on the  $\alpha^{\text{HE}}$  mutation. The incidence of the  $\alpha^{\text{V}/41}$  allele is dramatically high, affecting nearly one chromosome out of three in Caucasians. Beside the  $\alpha^{\text{V}/41}$  allele,  $\alpha^{\text{LE}}$  alleles of a different kind have been described (16, 17).

In the present work, we further describe the  $\alpha^{\text{V}/41}$  allele. The  $\alpha^{\text{V}/41}$  phenotype was attributed to a mutation affecting nucleotide (nt) 7 of exon 40. The synthetic defect was hypothetically related to partial skipping of exon 46. In addition, a mutation affecting nt-12 of intron 45 was recorded. So far, mutations in exon 40 and intron 45 have always been found in *cis* of one another, defining allele  $\alpha^{\text{LELY}}$  (LE, LYon). Whenever appropriate, this designation will gradually replace the previous  $\alpha^{\text{V}/41}$  terminology.

## Methods

**Conventions.** The mutation responsible for the peptide maps' abnormalities (increase of the  $\alpha\text{V}$  41-kD fragment) will be referred to as the exon 40 mutation (or the  $\alpha^{\text{V}/41}$  mutation). The mutation in intron 45 will be designated intron 45 mutation. The 5' region, encoding the  $\alpha\text{I}$  and  $\alpha\text{II}$  domains, carries all presently elucidated  $\alpha$ -chain HE mutations (+, presence; -, absence). The 3' region, encoding the  $\alpha\text{IV}$  and  $\alpha\text{V}$  domains, bears the exon 40 and intron 45 mutations (-, absence; +, presence). Altogether, haplotypes will be defined on the basis of these three markers (HE mutation, exon 40 mutation, and intron 45 mutation), written in the 5'  $\rightarrow$  3' direction. Four haplotypes were encountered: +--, ---, -++ and, in one family, +++.

**Kindreds.**  $\alpha^{\text{V}/65}$  HE family BE (12, 14, 18) (Table I) was chosen for

1. **Abbreviations used in this paper:** HE, hereditary elliptocytosis; LE, low expression; nt, nucleotide; RT, reverse transcription.

the search of the  $\alpha^{V41}$  mutation because of a favorable genetic situation. Studies on proteins indicated the following diplotypes:  $+-\cdot/---$  (father, I.1),  $++\cdot/+-\cdot$  (mother, I.2), and  $+-\cdot/+-\cdot$  (propositus, II.1) ( $\cdot$ , initially undetermined status of intron 45 mutation). Other families (Table I) were families TR ( $\alpha^{I78}$  allele); NO and CH ( $\alpha^{I74}$  alleles); AK, AZ, BA, HD, RA, and TI ( $\alpha^{I65}$  allele); FE ( $\alpha^{II31}$  allele); and HA ( $\alpha^{II21}$  allele), as presented or referred to previously (14). Since this report, the responsible mutations were characterized in families NO (19), FE (20), and HA (20a). Mutations were also identified in additional families BL (21), BY (22), and SR (Dalla Venezia, N., N. Alloisio, A. Forissio, L. Denoroy, M. Aymerich, J. L. Vives Corrons, J. Besalduch, I. Besson, and J. Delaunay, manuscript submitted for publication). In family JO, the mutation stands in the  $\alpha$ -chain but remained unidentified.

**cDNA analysis.** Reticulocyte RNA preparation, reverse transcription (RT), and polymerase chain reaction (PCR) were carried out essentially as described or referred to previously (22). The PCR mixture was adjusted to 100  $\mu$ l so as to contain 20 mM Tris HCl (pH 8.40), 50 mM KCl, 2.5 mM MgCl<sub>2</sub>, 50  $\mu$ M dNTP, and 0.01% gelatin. The  $\alpha$ V domain cDNA was amplified according to two segments that overlapped at the level of exon 43.

The segment extending from exons 38 to 43 was amplified (Fig. 1; Table II) (primers, A/B; 30 cycles; denaturation, 92°C, 60 s; annealing, 56°C, 30 s; extension, 72°C, 60 s). Sequences of primers are given in Table II. The amplified 625-bp fragment (see below) was cloned as a BamHI insert in phage M13mp19 as described before (23) and sequenced.

The segment of  $\alpha$ V domain cDNA extending from exon 43 to a region located 3' of the VNTR (196 nt downstream of the TAG termination signal) was amplified (primers, C/D; 30 cycles; denaturation, 92°C, 60 s; annealing, 56°C, 30 s; extension, 72°C, 60 s). In view of analyzing the RT-PCR products of the  $\alpha$ V domain 3' moiety, we first considered the possibility of an exon skipping. This exon, however, had to be short enough not to alter the apparent length of the single 1,473-bp PCR product that was obtained (see below), as well as not to introduce a frameshift. As such, exon 46 seemed a likely candidate, especially since its skipping had been previously observed in human fibroblast nonerythroid  $\alpha$ -spectrin (24). With PvuII sites standing 5' and 3' of exon 46 on cDNA, the 1,473-bp fragment was digested with this enzyme. Later on, skipping of exon 46 was monitored directly using RT-PCR and primers M and J. We obtained fragments with the following sizes: 155 bp (exon 46 present) and 137 bp (exon 46 absent) (see

Table I. The Families

$\alpha^{I78}$ ; 41 Arg $\rightarrow$ Trp				I.2*	---/-++
TR	I.1*	---/-++		II.5*	+++/-++
	I.2	++-/---		II.6*	+++/-++
	II.7*	++-/---		II.8	+++/----
				II.12*	+++/-++
$\alpha^{I74}$ ; 28 Arg $\rightarrow$ His (19)			$\alpha^{I50-46a}$ ; 207 Leu $\rightarrow$ Pro <sup>a</sup> (21)		
NO	I.1	+++/----	BL	I.1*	+++/----
	I.2 <sup>‡</sup>	+++/-++		I.2*	+++/----
	II.1*	+++/-++		II.2 <sup>‡</sup>	+++/-++
$\alpha^{I74}$ ; 46 Gly $\rightarrow$ Val			$\alpha^{I36}$ ; activation of a donor splice site (22)		
CH	I.1	+++/----	BY	I.1*	+++/-++
	I.2*	+++/-++		I.2*	+++/-++
	II.2*	+++/-++		II.1	+++/-++
$\alpha^{I65}$ ; 154 + Leu			$\alpha^{I50-46b}$ ; 469 His $\rightarrow$ Pro (Dalla Venezia et al., manuscript submitted for publication)		
AK	I.1	+++/----	SR	I.1*	+++/-++
	I.2*	+++/-++		I.2	+++/----
	II.9*	+++/-++		II.1*	+++/-++
AZ	I.1	+++/----	$\alpha^{II31}$ ; 791 Asp $\rightarrow$ Glu (20)		
	II.1	+++/----	FE	I.1 <sup>‡</sup>	+++/-++
	II.2	+++/----		I.2	+++/----
BA	I.1	+++/----		II.1*	+++/-++
	I.2	+++/----	$\alpha^{II21}$ ; skipping of exon 18 (20a)		
	II.1	+++/----	HA	I.1	+++/----
	II.2	+++/----		I.2*	+++/-++
BE	I.1	+++/----		II.1*	+++/-++
	I.2 <sup>‡</sup>	+++/-++		II.2	+++/-++
	II.1*	+++/-++	$\alpha^{HE}$ ; unidentified		
HD	I.1*	++-/+-	JO	I.1	+++/-++
	I.2	++-/---		I.2	+++/----
RA	I.2	+++/----		II.2	+++/-++
	II.1*	+++/-++			
	II.2*	+++/-++			
TI	I.1	+++/----			

Amino acids in spectrin  $\alpha$  chain are numbered according to Sahr et al. (7). Many of the HE mutations have been recapitulated before (14). References are explicitly provided only for recently characterized  $\alpha^{HE}$  mutations, especially in families not presented in reference 14. \* Heterozygosity and <sup>‡</sup> homozygosity for the  $\alpha^{V41}$  polymorphism on the basis of the percentage of the  $\alpha$ V 41-kD fragment, as reported in the various articles referred to. Each haplotype is defined by three signs. Left sign, absence (-) or presence (+) of the  $\alpha^{HE}$  mutation (peptide maps and/or DNA analysis). Middle sign, absence (-) or presence (+) of the exon 40 mutation (DNA analysis). Right sign, absence (-) or presence (+) of the intron 45 mutation (DNA analysis).  $\cdot$ , Presence or absence of the intron 45 mutation not examined. a,  $\alpha^{HE}$  mutation on a  $\alpha^{LELY}$  allele.

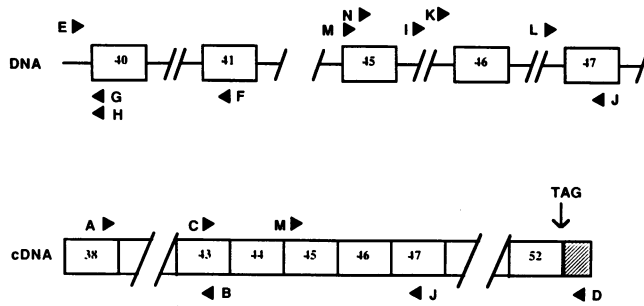


Figure 1. Positioning of oligonucleotides. Oligonucleotides P and Q do not appear (see Table I).

below). The 1,473-bp fragment was also sequenced in extenso after cloning as an EcoRI insert in phage M13mp18.

**Genomic DNA analysis.** Screening of exon 40 mutation was based, essentially, on dot blot hybridization (primers, E/F; ASO, G/H) (Table II). Primer E, as well as primers I, K, and L (see below), were designed according to unpublished sequences. The ASOs were labeled with digoxigenin and hybridization was detected immunochemically (25). In some cases, we used the PCR-mediated restriction site modification method (not shown). A genomic DNA segment extending from the 5' region of intron 45 to exon 47 was PCR amplified (primers, I/J) and submitted to direct sequencing (primers K or L) (Table II). A larger genomic DNA segment extending from exon 45 to 47 was also PCR amplified (primers, M/J) and submitted to direct sequencing (primer N). Screening of intron 45 mutation was on the basis of the abolishment of an *Ava*II site (occurrence of a 840-bp fragment vs. 318-

Table II. Oligonucleotides

A	CA (GGATCC) TCAAAGAGTTGGCCAAGGCC *
B	CA (GGATCC) GCGGCATAACGCTCTTCAAT †
C	GC (GAATTC) TTGAAGAGCGTTATGCCGCT *
D	GC (GAATTC) ATAGCTTCCACTCTCCAAC †
E	ctgtagctctgaatatgagcg *
F	ATTCAGAGCCTCTATCTTGG †
G (ASO)	GCTTCATTAGCAAGCTctg †
H (ASO)	GCTTCATTACCAAGCTctg †
I	catctgacagaggcagagaa *
J	TCCAGCTGAGATTCCAGAGT †
K	tctgaggaatgagatc *
L	ggagcttcgagttgga *
M	GCTGCAAAAGGAAGAGGCAA *
N	GATGTGTCAGGAGTTTG *
P	AACCGTTGTGGAGAGCAGTG *
Q	GGTCTTCATAGCTCTTATCGG †

\* Sense, † Antisense. Intronic sequences appear in lower-case characters. Primers A (exon 38), B (exon 43), C (exon 43), and D (196 nt downstream of the TAG termination signal) were used for the PCR amplification of the  $\alpha^V$  41-kD domain cDNA. Primers E (intron 39) and F (exon 41), amplification of the genomic DNA encompassing exon 40. ASO G and H (intron 39/exon 40), screening of controls and individuals as for exon 40 mutation. M (exon 45) or I (intron 45) and J (exon 47), amplification of the genomic DNA extending from exon 45 or intron 45 to exon 47. Primers M and J, RT-PCR amplification encompassing exon 46. N (exons 45), K (intron 45), and L (intron 46), direct sequencing of the exon 45/intron 45, intron 45/exon 46, exon 46/intron 46, and intron 46/exon 47 boundaries. P and Q (not represented in Fig. 1), RT-PCR around position  $\alpha 28$  in family NO. Primers E, I, K, and L were designed according to unpublished sequences.

and 528-bp fragments (sizes determined on gel), after amplification with primers I/J and digestion with *Ava*II). Screening of a mutation found in intron 46 (see below) was on the basis of the creation of a *Taq*I site (occurrence of 58- and 56-bp fragments vs. a 114-bp fragment (sizes determined on gels), after amplification with primers I/J and *Taq*I digestion).

**Estimation of mRNA level.** Total mRNA produced by the  $\alpha^{V/41}$  allele was determined. In family NO, RT-PCR was carried out (primers, P/Q; 30 cycles; denaturation, 92°C, 60 s; annealing, 57°C, 30 s; extension, 72°C, 60 s). The  $\alpha^{HE}$  mutation abolished a *Bsa*HI site: 230-bp fragment vs. 169- and 61-bp fragments (theoretical sizes). In family BY, RT-PCR was performed using primers that had been designated "A" and "B" in a previous work (22) (30 cycles; denaturation, 92°C, 60 s; annealing, 57°C, 30 s; extension, 72°C, 60 s). 296- and a 269-bp fragments (theoretical sizes) represented total non-HE mRNAs (arising from the  $\alpha^{V/41}$  allele (see below)) and the  $\alpha^{HE}$  mRNA, respectively (the difference was due to the splicing out of 27 nt in the 3' region of exon 8). DNA fragments were estimated densitometrically using ethidium bromide or autoradiography ( $[^{35}\text{S}]\text{dATP}$ ) depending on the experiments.

mRNA  $\alpha^{LELY}$  was also determined. In a strict sense, mRNA  $\alpha^{LELY}$  represents only a fraction of the mRNA that arises from allele  $\alpha^{LELY}$ , e.g., the fraction that lacks exon 46 (see below). Reticulocyte mRNA from individuals BE I.1, BE I.2, and BY II.1 was RT-PCR amplified using  $[^{35}\text{S}]\text{dATP}$  labeling. 1  $\mu\text{l}$  of  $[^{35}\text{S}]\text{dATP}$  (10  $\mu\text{Ci}$ ) was added to the PCR mixture. The latter was submitted to 23 cycles (primers M/J; denaturation, 92°C, 60 s; annealing, 59°C, 30 s; and extension, 72°C, 30 s). Preliminary experiments using variable numbers of cycles showed that a 23-cycle procedure was in the interval of proportionality between the amount of amplified DNA and the amplification time (data not shown). cDNA was monitored electrophoretically and estimated by densitometry after autoradiography. 155- and 137-bp fragments were obtained (see below). The 137-bp fragment corresponded to mRNA  $\alpha^{LELY}$  (exon 46 absent).

## Results

**Exon 40 mutation.** RT-PCR (individual BE I.2; primers, A/B) yielded a 625-bp fragment (theoretical size). Sequencing of this fragment disclosed the C  $\rightarrow$  G substitution (CTA  $\rightarrow$  GTA) at the level of nt 7 of exon 40 (Fig. 2). This change yields the Leu  $\rightarrow$  Val substitution at position 1,857, e.g., at position 69 (helix 2) of repeating segment  $\alpha 18$ . It was previously mentioned as one difference between cDNA (CTA) (6) and genomic DNA (GTA) (8).

61 chromosomes from 16 families were screened for the presence or the absence of exon 40 mutation (Table I). With no single exception, this mutation appeared when the  $\alpha^{V/41}$  polymorphism was present (19 chromosomes) and failed to

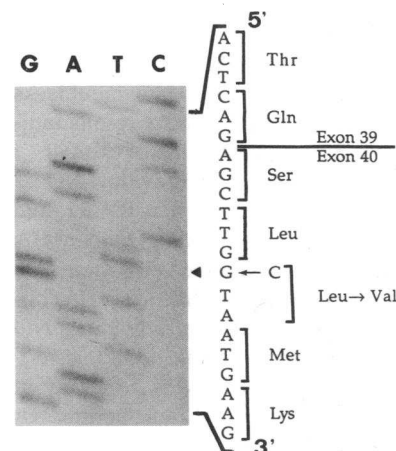
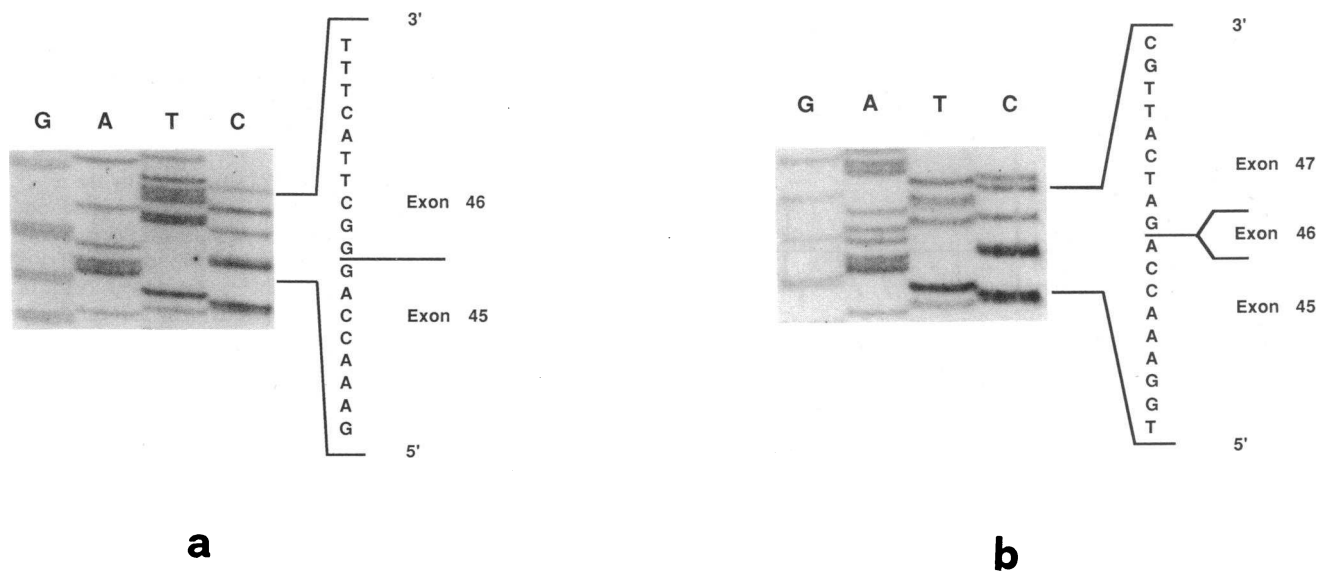


Figure 2. Exon 40 mutation. In individual BE I.2 ( $\alpha^{V/41}/\alpha^{V/41}$  homozygote), the C  $\rightarrow$  G substitution occurred at position 7 of exon 40 (CTA  $\rightarrow$  GTA;  $\alpha 1857$  Leu  $\rightarrow$  Val).



**Figure 3.** Nucleotide sequencing. cDNA was amplified (primers, C/D; individual BE I.2) into a 1,473-bp fragment that was thereafter digested with PvuII. (a) The 345-bp fragment contains exon 46. (b) The 327-bp fragment lacks exon 46.

appear when this polymorphism was absent (42 chromosomes).

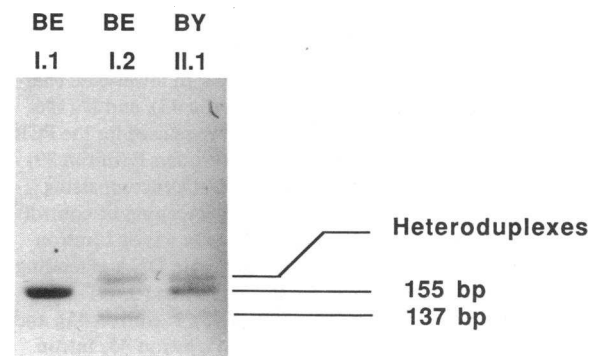
Tentative estimation of total mRNA arising from the  $\alpha^{V/41}$  allele indicated that the latter was not less represented than the  $\alpha^{HE}$  mRNA. In family NO, the 169- and 61-bp fragments (total mRNA arising from the  $\alpha^{V/41}$  allele) amounted to the 250-bp fragment ( $\alpha^{HE}$  mRNA) (not shown). In family BY, the 296-bp fragment (total mRNA arising from the  $\alpha^{V/41}$  allele) also amounted to the 269-bp fragment ( $\alpha^{HE}$  mRNA), the presence of heteroduplexes being taken into account (not shown). These estimations made it unlikely that the quantity of mRNA produced by the  $\alpha^{V/41}$  allele would be severely diminished, as is the case for mRNAs arising from other  $\alpha^{LE}$  alleles (16, 17).

**Partial skipping of exon 46.** It appeared convincing at this point that exon 40 mutation would account for the structural changes ( $\alpha^{V/41}$  phenotype). It was not sure, however, whether it would explain the low expression character of this allele. RT-PCR (individual BE I.2; primers, C/D) yielded a 1,473-bp fragment (theoretical size). Nucleotide sequencing of the overall (1,473-bp) fragment disclosed clones with exon 46 and clones without exon 46 (Fig. 3). After PvuII digestion of this fragment, the 345-bp subfragment appeared duplicated into a 327-bp fragment (theoretical sizes) (not shown). Comparable results were obtained after RT-PCR using primers M and J (individual BE I.2). The normal 155-bp fragment was duplicated into a 137-bp fragment (theoretical sizes) (Fig. 4). Nucleotide sequencing established that the 137-bp fragment lacks exon 46 (not shown). Taken together, the above results indicated that the  $\alpha^{V/41}$  allele yields partial skipping of exon 46.

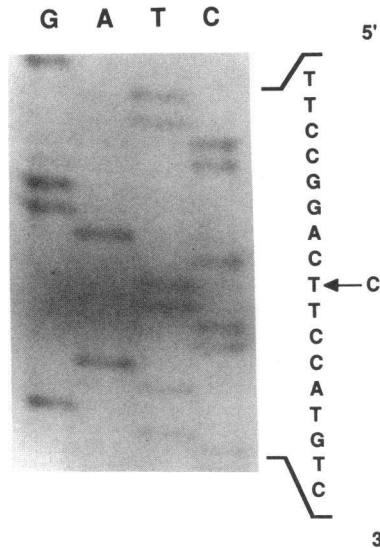
Skipping of exon 46 was also observed in individual NO I.2 ( $\alpha^{V/41}$  homozygote) and in individuals NO II.1, BY I.1, and II.1, JO II.2, and HA I.1' ( $\alpha^{V/41}$  heterozygotes) (individual HA I.1' is not presented in Table I but is described elsewhere (15; Alloisio et al., manuscript submitted for publication)). In contrast, no skipping of exon 46 was recorded in individuals NO I.1, BE I.1, BY II.2, HA I.2', and II.1' ( $\alpha/\alpha$ ) (individuals BY II.2 (22), HA I.2', and II.1' (15; Alloisio et al., manuscript submitted for publication) do not appear in Table I either but are described in the indicated references).

The extent of exon 46 partial skipping was determined using a 23-cycle RT-PCR amplification (primers, M/J), a number of cycles standing in the interval of proportionality (see Methods). The 137-bp fragment (exon 46 absent) accounted for 50% of all RT-PCR products, e.g., the sum of this fragment and the 155-bp (exon 46 present) fragment in homozygous individual BE I.2 ( $\alpha^{V/41}/\alpha^{V/41}$ ). It accounted for 25% of total products in heterozygous individual BY II.1 ( $\alpha/\alpha^{V/41}$ ). Altogether, allele  $\alpha^{V/41}$  gives rise to two mRNA subspecies in a roughly equivalent amount: mRNA  $\alpha^{V/41}$  (exon 40 mutation, present; exon 46, present) and mRNA  $\alpha^{LELY}$  (exon 40 mutation, present; exon 46, absent). In turn, the sum of these two subspecies accounted for 50% of total mRNAs in a  $\alpha/\alpha^{V/41}$  heterozygote (mRNA  $\alpha^{LELY}$ , ~ 25%; mRNA  $\alpha^{V/41}$ , ~ 25%). This figure, completing data presented above, indicates that allele  $\alpha/\alpha^{V/41}$  is not associated with a dramatic reduction of mRNA production.

**Intron 45 mutation.** We sequenced the following exon-intron boundaries: exon 45/intron 45, intron 45/exon 46, and exon 46/intron 46 (individuals BE I.1, I.2, and II.1), and intron 46/exon 47 (individuals NO I.1, I.2, and II.1). We found a substitution (C  $\rightarrow$  T) at nt-12 of intron 45 (in comparison



**Figure 4.** RT-PCR amplification (primers, M/J). The normal 155-bp fragment was duplicated into a 137-bp fragment. BE I.1,  $\alpha/\alpha$ ; BE I.2,  $\alpha^{V/41}/\alpha^{V/41}$ ; BY II.1,  $\alpha/\alpha^{V/41}$ .



**Figure 5.** Intron 45 mutation. Genomic DNA was amplified (primers, I/J; 840-bp fragment [determined size]) in individual BE I.2 ( $\alpha^{V/41}/\alpha^{V/41}$  homozygote). Nucleotide sequencing disclosed a substitution (T  $\rightarrow$  C) at position -12 of intron 45.

with an unpublished sequence) (Fig. 5). In addition, there was a substitution (G  $\rightarrow$  A) at nt-12 of intron 46 (not shown) in all  $\alpha^{V/41}$  chromosomes.

Screening the intron 45 mutation in 47 individuals (53 chromosomes) indicated the presence or the absence of this mutation whenever the exon 40 mutation was present or absent, respectively (Table I). The mutation of intron 46 was present in all individuals carrying the exon 40 and intron 45 mutations. However, it was also present in some individuals (BE I.1, FE I.2, and CH I.1) among investigated individuals who were devoid of exon 40 and intron 45 mutations. In individual AK I.2 the mutation in intron 46 occurred in the homozygous state whereas exon 40 and intron 45 mutations were only present in the heterozygous state. Altogether, intron 45 mutation is exclusively linked to exon 40 mutation (with no single exception so far). The mutation in intron 46 always occurs in *cis* of exon 40 and intron 45 mutations but happens, in addition, on some  $\alpha$  alleles.

## Discussion

We have further characterized a LE  $\alpha$  allele previously referred to as allele  $\alpha^{V/41}$ . This allele has been renamed allele  $\alpha^{LELY}$ . Its structural defect, the increased proteolytic susceptibility of the  $\alpha IV$ - $\alpha V$  domain junction, can be related to exon 40 mutation. This mutation occurs in helix 2 of repeating segment  $\alpha 18$  (po-

sition 69) whereas the sensitized cleavage lies in helix 3 (between positions 23 and 24) of repeating segment  $\alpha 19$  (WK/LQ). According to the proposed triple-helical model of spectrin chain structure in space (26, 27), helices 2 and 3 are adjacent. Within the same conformational unit, a mutation lying in helix 2 is likely to induce a conformational change in its neighbor helix 3. Comparable situations have been encountered before in HE (28–32). One may wonder why the exon 40 mutation does not itself yield HE. Despite a similar spatial organization, the external regions of the spectrin tetramer would not have the same function as its central region. The external regions participate in the nucleation of dimer assembly (33). The central region is involved in dimer self-association. All known HE mutations of spectrin occur between repeating segments  $\beta 17$  and  $\alpha 9$ , as has been discussed (20).

Yet the synthetic defect associated with allele  $\alpha^{LELY}$  would not easily be accounted for by exon 40 mutation. The estimated level of total mRNA yielded by this allele ruled out, at least, a major transcriptional deficit or a gross posttranscriptional abnormality. In contrast, the synthetic defect might arise from the partial skipping of exon 46, a more limited posttranscriptional abnormality. Hypothetically, partial skipping of exon 46 could stem from intron 45 mutation even though this mutation, occurring in a pyrimidin-rich region (34), would not be expected to affect splicing. Additionally, it could be that intron 45 mutation is assisted in this role by the mutation in intron 46. The latter would have no effect by itself since it occurs on some normal  $\alpha$  alleles without aggravating the expression of the  $\alpha^{HE}$  allele located in *trans*.

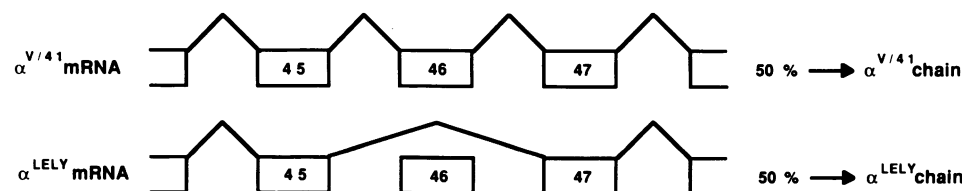
Exon 46 would play a role in the nucleation of dimer assembly. It could seem unlikely that such a small exon (18 nt) is important given the size of the nucleation site (33). On the other hand, one may argue that the conservation of exon 46 must reflect a critical function at least in some cell types.  $\alpha^{LELY}$  chains (mutation 40, present; exon 46, absent; see Fig. 6) would be incorporated little or not at all into the dimer and degraded. In this respect,  $\alpha^{LELY}$  chains would be associated, expectedly, with an increased turnover. There is a good correspondence between the estimated percentage ( $\sim 50\%$ ) of  $\alpha^{V/41}$  chain (exon 40 mutation, present; exon 46, present; see Fig. 6) incorporated into the membrane, as has been previously stated in detail (14, 15), and the estimated percentage of mRNA  $\alpha^{V/41}$  ( $\sim 50\%$  of total mRNA arising from allele  $\alpha^{LELY}$ ; this work).

Nonetheless, allele  $\alpha^{LELY}$  is able to provide the erythroid precursors with sufficient amounts of  $\alpha^{V/41}$  chains, hence, its

### Normal $\alpha$ allele



### $\alpha^{LELY}$ allele



**Figure 6.** Schematic representation of  $\alpha^{LELY}$  and  $\alpha^{V/41}$  mRNAs, both arising from allele  $\alpha^{LELY}$ .

innocuous character. Its key effect arises when it interacts, in *trans*, with an  $\alpha^{\text{HE}}$  allele. In the  $\alpha^{\text{HE}}/\alpha^{\text{LELY}}$  compound heterozygous state,  $\alpha^{\text{HE}}$  chains are normal in terms of dimer formation. Therefore, they compete advantageously with the products of allele  $\alpha^{\text{LELY}}$ , e.g., the  $\alpha^{\text{LELY}}$  chains assumed to undergo poor or no recruitment and the  $\alpha^{\text{V/41}}$  chains, the pool of which is reduced by 50% as we have said. Accordingly, a majority of  $\alpha^{\text{HE}}\beta$  dimers are synthesized. The erythroid precursor cells would then find themselves trapped by the large proportion of  $\alpha^{\text{HE}}\beta$  dimers, which are deficient in self-association due to the HE mutation; hence, the development of severe conditions.

Allele  $\alpha^{\text{LELY}}$  contains two linked mutations: the exon 40 mutation (or  $\alpha^{\text{V/41}}$  mutation), a presumably spectator mutation responsible for the  $\alpha^{\text{V/41}}$  structural changes, and the intron 45 mutation. It is associated with partial skipping of exon 46, a fact that may account for a defect in dimer assembly.

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