Hereditary Spherocytosis With Band 3 Deficiency. Association With a Nonsense Mutation of the Band 3 Gene (Allele Lyon), and Aggravation by a Low-Expression Allele Occurring in trans (Allele Genas)

By Nicole Alloisio, Philippe Maillet, Geneviève Carré, Pascale Texier, Agnès Vallier, Faouzi Baklouti, Noël Philippe, and Jean Delaunay

We describe an 18-year-old with moderate hereditary spherocytosis. The condition was associated with a 35% decrease in band 3. The underlying mutation was Arg to stop at codon 150 (CGA→TGA) and was designated R150X, which defined allele Lyon of the EPB3 gene. The inheritance pattern was dominant. However, the mother, who also carried the allele Lyon, had a milder clinical presentation and only a 16% decrease of band 3. We suggested that the father had transmitted a modifying mutation that remained silent in the heterozygous state. Nucleotide sequencing after single strand conformation polymorphism analysis of the band 3 cDNA and promoter region revealed a G→A substitution at position 89 from the cap site in the 5′-untranslated region, designated 89G→A, which defined allele Genas. A ribonuclease protection assay showed that (1) the allele Genas (father) resulted in a 33% decrease in the amount of band 3 mRNA, (2) the reduction caused by the allele Lyon (mother) was 42%, and (3) the compound heterozygous state for both alleles (proband) resulted in a 58% decrease. These results suggest that some mildly deleterious alleles of the EPB3 gene are compensated for by the normal allele in the heterozygous state. They are shown through the aggravation of the clinical picture, based on more obvious molecular alterations when they occur in trans to an allele causing a manifest reduction of band 3 membrane protein concentration.

© 1996 by The American Society of Hematology.

From the Laboratoire de Génétique Moléculaire Humaine, CNRS URA 1171, Institut Pasteur de Lyon, Lyon; and Hématologie Pédiatrique, Hôpital Debroussé, Lyon, France.

Supported by the Université Claude-Bernard Lyon-I; the Centre National de la Recherche Scientifique (URA 1171 and PICS 221, a joint program with the Ministère des Affaires Étrangères, Paris, France, and Monbusho, Tokyo, Japan); the Institut National de la Santé et de la Recherche Médicale (CRE 930405); the Conseil Régional de la Région Rhône-Alpes; the Association Française contre les Myopathies; the Fondation Pour la Recherche Médicale; the Association de Recherche contre le Cancer; the Programme Hospitalier de Recherche Clinique (1995-1997); and the Institut Pasteur de Lyon.

Address reprint requests to Nicole Alloisio, PhD, Laboratoire de Génétique Moléculaire Humaine, CNRS URA 1171, Institut Pasteur de Lyon, avenue Tony Garnier, 69365 Lyon Cedex 07, France.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1996 by The American Society of Hematology. 0006-4971/96/8803-0003$00/0

Blood, Vol 88, No 3 (August 1), 1996; pp 1062-1069
BAND 3 DEFICIENCY IN HEREDITARY SPHEROCYTOSIS

Table 1. Routine Hematological Data

<table>
<thead>
<tr>
<th></th>
<th>RBC T/L (4-5.5)</th>
<th>Hb g/L (115-165)</th>
<th>Ht L/L (0.38-0.52)</th>
<th>MCV fL (85-100)</th>
<th>Reticulocytes G/L (10-40)</th>
<th>Bilirubinemia μmol/L (&lt;20)</th>
<th>Serum Haptoglobin g/L (&gt;0.4)</th>
<th>'Pink Test' % (&lt;16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Father</td>
<td>5.3</td>
<td>155</td>
<td>0.49</td>
<td>91</td>
<td>67</td>
<td>7</td>
<td>0.6</td>
<td>31</td>
</tr>
<tr>
<td>Mother</td>
<td>4.6</td>
<td>139</td>
<td>0.42</td>
<td>91</td>
<td>277</td>
<td>25</td>
<td>0.1</td>
<td>265</td>
</tr>
<tr>
<td>Proband*</td>
<td>3.3</td>
<td>102</td>
<td>0.30</td>
<td>91</td>
<td>413</td>
<td>32</td>
<td>0.04</td>
<td>935</td>
</tr>
</tbody>
</table>

Normal ranges are indicated in parentheses.

Abbreviations: RBC, red blood cells; MCV, mean corpuscular volume.

* Red cell osmotic resistance test described by Vettore et al. (the results are expressed as the percentage of hemolysis with respect to total hemolysis). The greater fragility of the proband’s red cells compared with the mother’s red cells was confirmed with a second blood sample in 1994 (not shown).

† 1987.
‡ 1986.
§ 1993.

was negative. Bone marrow showed an increased number of white cell precursors and a pronounced erythroid blastosis. At that time, the patient received two transfusions and corticosteroid therapy. Two months later, the routine laboratory data (see Table 1) showed hyperbilirubinemia, decreased serum haptoglobin, and hyperreticulocytosis. During the following months, the hemoglobin concentration fluctuated between 103 and 132 g/L and the reticulocyte cell count between 140 and 300 g/L. In 1991, scleral icterus reappeared when the patient presented asthenia, hyperthermia and abdominal pains and, in 1993, stones were detected in the biliary tract, along with gastritis. The ‘Pink test’ revealed a strong decrease of red cell osmotic resistance (see Table 1). Early in 1995, the child developed obstructive jaundice that resolved spontaneously. Echography revealed a 9 mm stone in the gall bladder and a cluster of infundibular microstones. The spleen was 2 cm below the costal margin. Cholecystectomy was performed in April 1995.

The father (a simple heterozygote for allele Genas; discussed later) was born in 1952. He is symptomless and has normal red cell data (Table 1). The mother (a simple heterozygote for allele Lyon; discussed later) was born in 1954. She had no clinical symptoms and no history of hyperhemolysis but routine laboratory data, obtained in 1987, revealed compensated hemolysis (Table 1). The osmotic resistance of red cells was low and some spherocytes were visible on blood smears (Fig 1).

MATERIALS AND METHODS

Densitometric Quantitation of Erythrocyte Membrane Proteins

Red cell membrane proteins were analyzed by polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS-PAGE), using a 5% to 15% linear gradient or 3.5% to 17% exponential gradient, and scanned at 570 nm using a System II Densitometer (Sebia, Issy-les-Moulineaux, France). The control values are given as the mean ± SD.

Studies on EPB3 Gene

DNA was extracted from white blood cells. The promoter region and the capping site located in exon 1 of the EPB3 gene were amplified with primers Q/R, S/T, and the exons 4 and 6 with primers L/M and N/O, respectively. (Primers were designed according to Schofield et al. and Tannert et al [unpublished data] (Table 2)). The polymerase chain reaction (PCR) product of exon 6, which was too large for single strand conformation polymorphism (SSCP) screening, was purified with the Prep-A-gene DNA Purification Kit (Bio-Rad, Hercules, CA) and digested with BamHI. SSCP analysis was performed as described by Spinardi et al with some modifications. Briefly, PCR products and digested fragments were heat-denatured in five volumes of 0.05% SDS, 15 mmol/L EDTA, 47.5% formamide, 0.025% xylene, 0.025% bromophenol.

Electrophoresis was performed on a Hydrolink MDE gel (Bioprobe Systems, Montreuil-sous-Bois, France) in a Bio-Rad Protean II xi cell at 480 V for 2.5 hours with circulating water. PCR products of exon 6 (primers N/O) of the mother and the proband (containing the abnormal SSCP fragment) were subcloned into the pCR II vector (TA cloning Kit; Invitrogen, San Diego, CA). Ten subclones from each patient were PCR amplified (primers N/O) and screened by SSCP to distinguish normal from mutated sub-

Fig 1. Blood smears. M, mother; P, proband. Spherocytosis is more pronounced in the proband.
Table 2. Oligonucleotides Used as Primers

| Band 3 primers: | AAGGCAAGGAGCAACCTGAGTG* (−148−129) |
|                | GGGGCAACCTTCTTCTGCAAGG* (698−610) |
|                | CACGAGCCTTCTTCGGAGCA* (888−808) |
|                | TCCGACACCTCCCTCAGTGTTT* (1318−1297) |
|                | AGATCCAGCTTCCACAGGG* (1062−1081) |
|                | GGTGGACACCTGAGGACCATG* (1528−1507) |
|                | TATCACTCCTCGAGCTTCC* (25−6) |
|                | CACCATGAATGTTCTCGCG* (intron 1) |
|                | CACCATGACAGTGG* (intron 2) |
|                | CTACCTCCATGGCGACTGA* (intron 3) |
|                | TCCACTCTCATGCTGCTG* (intron 4) |
|                | TAGAGCGACGGAGCTTGGAC* (323−342) |
|                | ACTAGCAGATGAGCTTGGA* (intron 6) |
|                | PCCACACATCCATGCGATC* (intron 5) |
|                | AGTGGGACAGGAGCTTGGAC* (−502 to −481 of exon 1) |
|                | RGGGCACACAGGGGAGCTTG* (−202 to −221 of exon 1) |
|                | SACCCACAGTGGCGCTTGGAC* (−252 to −231 of exon 1) |
|                | TGGTGAGCTGCGTAGAGTCTTG* (1867−1867) |
|                | TGGCACCAGAATCTGCAGTTT* (2815−2798) |
|                | CAGAATCCACCTCGAGCTTCC* (25−6) |
|                | CTTACTGACTGAGCAGTGG* (intron 1) |
|                | ACTAGCAGATGAGCTTGGA* (intron 2) |
|                | AGTGGGACAGGAGCTTGGAC* (intron 3) |

Intronic sequences appear in lowercase characters. Letters in brackets correspond to a polycloning site sequence, complementary between primers U and W. Exonic band 3 primers were numbered according to Tanner et al. Intronic band 3 primers were designed according to Schofield et al. Exonic protein 4.1 primers were designed as previously described. Mutated nucleotide for creating a PCR-mediated restriction site.

| Protein 4.1 primers: | VAGAGAAAGACTACGATGCTTGG* (2096−2115) |
|                     | WCATATGCAGCTCAGTTGTTTACATGCTGCGACTTACATCGATGCTTGGAC* (2491−2510) |

RESULTS

Protein Analysis

Band 3 in the mother and the proband was significantly decreased compared with controls irrespective of the protein used as the internal standard (Table 3; Fig 2). This reduction confirmed in a second sample was more pronounced in the proband (−35%) than in the mother (−16%). There was also an accompanying reduction in protein 4.2 (proband: 4.2; mother: −28%; mother: −13%). We have observed a decrease in protein 4.2, which is considered to be secondary, in 13 unrelated families with band 3 deficiency. The chimeric PCR product was cloned in the pCR II vector (Invitrogen). Then, anti-mRNA transcripts were produced using [α-32P]uridine triphosphate (~3,000 Ci/mmol; JCN, Costa Mesa, CA), in an in vitro transcription system (Riboprobe, Gemini II Core system; Promega, Madison, WI). The probe was hybridized with 2 μg of total reticulocyte RNA from each subject and digested with a RNase mixture (Ambion Inc, Austin, TX). The protected fragments were separated using a PAGE, and their quantities measured with a ‘Preference Ecran HR’ Densitometer (Sebia).
BAND 3 DEFICIENCY IN HEREDITARY SPHEROCYTOSIS

Table 3. Densitometric Measurement of Band 3 and Protein 4.2*

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Father</th>
<th>Mother</th>
<th>Proband</th>
</tr>
</thead>
<tbody>
<tr>
<td>Band 3/spectrin</td>
<td>0.99 ± 0.05†</td>
<td>0.93 (−6%)</td>
<td>0.83 (−16%)</td>
<td>0.64± (−35%)</td>
</tr>
<tr>
<td>Band 3/protein 4.1</td>
<td>6.7 ± 0.3†</td>
<td>6.1 (−9%)</td>
<td>5.6 (−16%)</td>
<td>4.4± (−34%)</td>
</tr>
<tr>
<td>Band 3/actin</td>
<td>7.0 ± 0.3†</td>
<td>6.8 (−3%)</td>
<td>5.8 (−17%)</td>
<td>4.6± (−34%)</td>
</tr>
<tr>
<td>Protein 4.2/spectrin</td>
<td>0.120 ± 0.006†</td>
<td>0.111</td>
<td>0.105 (−13%)</td>
<td>0.089± (−26%)</td>
</tr>
<tr>
<td>Protein 4.2/protein 4.1</td>
<td>0.88 ± 0.03†</td>
<td>0.87</td>
<td>0.80 (−9%)</td>
<td>0.61± (−31%)</td>
</tr>
<tr>
<td>Protein 4.2/actin</td>
<td>0.92 ± 0.04†</td>
<td>0.90</td>
<td>0.83 (−10%)</td>
<td>0.64± (−30%)</td>
</tr>
</tbody>
</table>

* The spectrin ratios (band 3/spectrin and protein 4.2/spectrin) were calculated on gels according to Fairbanks et al. All other ratios were calculated on gels according to Laemmli. The band 3 and protein 4.2 to ankyrin 2.1 ratios were not used due to the increase in ankyrin 2.1 associated with high reticulocyte counts.

† n = 4.
‡ n = 6.
§ and ‖ Significant decrease compared with the control (P < .01 and P < .05, respectively).

Nucleic Acid Analysis

Nonsense mutation at position 150. We initiated SSCP analysis of the EPB3 gene by screening the exons 4 and 6 (because we already had primers for amplification of these two exons). PCR amplification yielded 336 and 442 nt fragments, respectively. The latter fragment, too long for SSCP screening, was digested using BamHI into (1) a 339 nt fragment that encompassed exon 6 and some of the flanking introns, and (2) a 103 nt fragment further along intron 6. SSCP analysis disclosed normal patterns for exon 4 and complicated patterns for exon 6, suggesting the presence of a polymorphism in some controls, the father, and the mother, and the existence of a mutation in the mother and the proband (Fig 3). Nucleotide sequencing showed that there was a mutation in exon 6 and a silent polymorphism in intron 5.

Mutant clones from exon 6 of the mother and the proband exhibited a change: R150X; CGA → TGA (Fig 4). The corresponding allele, designated the allele Lyon, abolished an AvaII site. After RT-PCR and AvaII digestion, all the RT-PCR products (316 nt) appeared to be completely digested into two fragments (194 and 122 nt) in the controls as well as in the mother and the proband (Fig 4) suggesting that mRNA Lyon was undetectable.

Two normal clones from the mother exhibited a 500-69C → T single base polymorphism in intron 5 (not shown), not present in the normal clone from the proband. This polymorphism was screened at the genomic level using a PCR-mediated restriction site procedure (see Materials and Methods). It was present in the father and the mother in the heterozygous state and was absent in the proband (not shown). Screening 32 unrelated controls showed that this silent intronic polymorphism is common (16 chromosomes in 64) (not shown).

Mutation at position 89. Band 3 cDNA was screened for SSCP from nt −148 upstream from the ATG initiation

![Figure 2](https://example.com/figure2.png)

**Figure 2.** SDS-PAGE of membrane proteins using a 5% to 15% linear gradient of acrylamide. C, control; F, father; M, mother; P, proband. (†), slight decrease of the band 3 and protein 4.2. (‖), pronounced decrease of the band 3 and protein 4.2 (see Table 3). The proportion of the two bands in the protein 4.1 doublet in the mother and the proband is due to reticulocytosis and does not affect the total amount of the two protein 4.1 components.
In the family members, in addition to this polymorphism, a SSCP (4) appeared in M and P, and this was due to a nonsense mutation in exon 6 (see Fig 4).

![SSCP analysis of exon 6 and the flanking intronic segments.](image)

Codon to nt 79 downstream from the TGA termination codon in the mother and the proband (failing to display mRNA Lyon). The amount of mRNA available in the father was too small and was kept for mRNA quantitation. The mother contained a normal pattern, but the proband showed a SSCP in addition to this polymorphism. In the family members, the amount of mRNA available in the mother was lower in the father (Table 3). As expected, total band deficiency in the membrane varied with the three subjects IC, M, and P, indicating that mRNA Lyon was not detectable in M and P.

**DISCUSSION**

The present case of HS is associated with band 3 deficiency caused by the absence of one haploid set of band 3 protein. This is due to none, or at least undetectable amounts, of the corresponding mRNA. The genomic change: R150X; CGA → TGA, defines the allele Lyon, a new allele of the EPB3 gene. Recently, two groups reported two other nonsense mutations of the EPB3 gene, suggesting that mRNA instability is the cause of these cases of HS with band 3 deficiency. In many other diseases, it has been repeatedly observed that nonsense mutations cause the variant mRNA to be highly unstable.

Such a clear-cut abnormality could define a homogeneous sub-subset of HS, particularly within families, because the deleterious allele results in no incorporation of band 3 in the membrane protein.
membrane. This assumption of homogeneity implies that the band 3 allele located in trans yields a constant (yet insufficient) compensation. These results show that this is not necessarily so.

The allele Lyon yielded a mild form of HS associated with band 3 deficiency. The allele Genas was clinically silent in the simple heterozygous state. The compound heterozygous state resulted in noticeable aggravation of the clinical presentation compared with that found with the allele Lyon in the simple heterozygous state. The amounts of band 3 incorporated in the membrane and band 3 mRNA were also lower in the compound heterozygous state.

A 33\% reduction of total mRNA in the father (Genas/normal) had no significant effect on band 3 protein incorporation. Above this threshold of 33\%, the reduction of membrane-inserted band 3 protein was proportional to the decrease in band 3 mRNA (band 3 membrane protein/band 3 mRNA: \(\sim 1.5\)) (Table 4). These results suggest that there would be a 50\% excess of band 3 synthesized. Further experiments will be necessary to confirm this.

cDNA Genas and the genomic DNA promotor region were screened for SSCP, but we cannot rule out the possibility that there was another mutation responsible for the lower amount of mRNA Genas, which was not detected by the SSCP technique. On the other hand, some mutations in the 5'-UT portion of \(89G\rightarrow A\) have been shown to be associated with a decrease in mRNA. The importance of the 5'-UT sequence to mRNA stability has been also suggested by several other studies.

From a clinical point of view, deleterious band 3 alleles, such as the allele Lyon, may unmask silent alleles of EPB3 gene in trans and, in return, be aggravated by them. Such silent alleles might be common, although none have so far appeared as widespread as the allele \(a^{ELY}\), a modifying factor of the spectrin \(a\)-gene related to hereditary elliptocytosis.

In this report, we described the interaction between two new alleles of the EPB3 gene. In the simple heterozygous state...
state, the allele Lyon, carrying a nonsense mutation and lacking the corresponding mRNA, yielded mild HS with band 3 deficiency. The allele Genas, bearing a mutation in the 5'-UT portion of the mRNA, did not produce any symptom although there was a 33% reduction in band 3 mRNA. In the compound heterozygous state, the allele Lyon could no longer compensate for the defect of the allele Genas, and hence lead to a noticeable aggravation of HS.

ACKNOWLEDGMENT

We thank the MA family for their cooperation; M.-A. Schreiner and D. Marmonier for their technical assistance; Dr M.J.A. Tanner for providing intronic sequences; N. Connan for the preparation of the report; and M.C. Haugh for checking the English wording and syntax.

REFERENCES


Hereditary spherocytosis with band 3 deficiency. Association with a nonsense mutation of the band 3 gene (allele Lyon), and aggravation by a low-expression allele occurring in trans (allele Genas)

N Alloisio, P Maillet, G Carre, P Texier, A Vallier, F Baklouti, N Philippe and J Delaunay