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Comparison of the Ankyrin $(AC)_n$ Microsatellites in Genomic DNA and mRNA Reveals Absence of One Ankyrin mRNA Allele in 20% of Patients With Hereditary Spherocytosis

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Combined deficiency of ankyrin and spectrin represents the most common biochemical abnormality in hereditary spherocytosis (HS). To examine whether a decrease in ankyrin mRNA represents a frequent cause of this type of HS, we took advantage of the reported $(AC)_n$ microsatellite polymorphism in the 3′ untranslated region of ankyrin cDNA. We first measured the number of AC repeats in genomic DNA encoding erythrocyte ankyrin in 36 unrelated Czech HS patients with combined ankyrin and spectrin deficiency and found 21 of these subjects (58%) to be heterozygotes for the $(AC)_n$ microsatellite size. Further analysis of reticulocyte RNA showed that ankyrin cDNA from 7 of these 21 heterozygotes (33%) contained only one of the two ankyrin alleles. We conclude that approximately ⅓ of ankyrin-deficient autosomal dominant HS is caused by reduced expression of one ankyrin allele which, in turn, is caused by either a reduced transcription of one allele of the mutated ankyrin gene or abnormal processing or decreased stability of the mutant ankyrin mRNA. Because ankyrin deficiency is detected in ≈60% of HS subjects, this result suggests that ≈20% of all HS is caused by a decreased expression of one ankyrin mRNA allele.

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HEREDITARY SPHEROCYTOSIS (HS) is a common inherited hemolytic anemia characterized by spheroidal shape of red blood cells (RBCs) that have a reduced cell surface area-to-cell volume ratio.[1] The disorder is clinically heterogeneous in terms of severity of hemolysis, which ranges from mild compensated hemolysis to severe hemolytic anemia, as well as inheritance pattern, which is both autosomal dominant, in the majority of cases, or autosomal recessive. The underlying genetic defects include mutations of α spectrin and β spectrin, the major proteins of membrane skeleton; band 3 protein, the major transmembrane protein, which serves as an anion exchanger; ankyrin, which anchors the spectrin-based skeleton to the red blood cell membrane via its binding to β spectrin and band 3 protein; and other defects.[1] Based on abnormalities in protein composition detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or direct quantitation of membrane proteins by radioimmunoassay, HS kindred can be divided into four major groups: (1) isolated deficiency of spectrin, (2) combined deficiency of spectrin and ankyrin, (3) deficiency of the band 3 protein, and (4) deficiency of the protein 4.2 or other less commonly found defects.[1,2]

The combined deficiency of spectrin and ankyrin, which was first reported in two subjects with a severe form of HS,[3] represents the most common biochemical phenotype.[4] The evidence indicating that the combined deficiency of spectrin and ankyrin results from a primary defect of ankyrin was established by studies of spectrin and ankyrin synthesis in erythroblasts and early reticulocytes.[5] In one of the patients with severe combined spectrin and ankyrin deficiency, these studies showed a marked decrease in ankyrin mRNA and ankyrin synthesis, leading to a markedly reduced incorporation of ankyrin into the RBC membrane. The spectrin mRNA and the spectrin synthesis were normal, or in the case of β spectrin, even increased, but the incorporation of both spectrin chains into the membrane skeleton and their steady-state levels were diminished in proportion to the reduced incorporation of ankyrin into the RBC membrane.[5] Several molecular defects of the ankyrin gene were reported to underlie this biochemical phenotype. These include a deletion of ankyrin gene on chromosome 8,[6] mutations within the regulatory domain of ankyrin that change alternative splicing of ankyrin (ankyrin^{PRAGUE}, ankyrin^{RAKOVNIK}),[7] and several frameshift or nonsense mutations.[8]

It has been documented that nonsense mutations in messenger RNAs often cause a reduction in the steady-state mRNA levels.[9] To examine whether ankyrin mutations leading to a decreased ankyrin mRNA expression represent a frequent cause of HS, we studied the previously described AC repeat polymorphism in the 3′ untranslated region of ankyrin cDNA.[10] We screened genomic DNA from 36 unrelated Czech patients with ankyrin-deficient HS and found 21 (58%) of them to be heterozygous for AC repeats of different sizes. We then analyzed reticulocyte RNA from the 21 heterozygotes and, in 7 (33%), detected only one of the two ankyrin alleles in the ankyrin cDNA. We conclude that, in the studied population, approximately ⅓ of ankyrin-deficient autosomal dominant HS is caused by an absence of one ankyrin mRNA allele. Because ankyrin deficiency is found in ≈60% of the studied HS subjects, these results suggest that ≈20% of all autosomal dominant HS is caused by a decreased expression of one ankyrin mRNA allele. Because the polymorphic dinucleotide and trinucleotide repeats are frequently found in the human genome,[11,12] the described approach of simultaneous detection of microsatellite sizes in genomic DNA and mRNA can be used to study the expression of not only erythroid ankyrin, but a number of other genes.

MATERIALS AND METHODS

Preparation of erythrocyte ghosts and quantitation of membrane proteins. Freshly drawn blood anticoagulated in acid citrate/dex-

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trose was shipped on ice from Prague to Boston. Within 48 hours of phlebotomy, erythrocyte ghosts were prepared by the method of Dodge et al[13] with minor modifications described in Jarolim et al.[14] Proteins were separated by SDS-PAGE in 3.5% to 17% exponential gradient gels according to Agre's modification[15] of the original Fairbanks' method[16] and in 12% Laemmli gels.[17]

Detection of the AC repeat polymorphism. Genomic DNA was isolated from 36 unrelated control subjects and polymerase chain reaction (PCR) amplified using primers P476 (5'-TCCCAGATC-GCTCTACATGA-3', nt 6276-6295) and P477 (5'-CACAGCTTC-AGAAGTCACAG-3', nt 6385-6366) [18] and the Perkin Elmer-Cetus (Norwalk, CT) GeneAmp PCR reagents kit (35 cycles, 1 minute at 95°C, 1 minute at 55°C) in the presence of P^{32}-deoxyadenosine triphosphate (dATP). The PCR products were electrophoresed in the BaseAce vertical sequencing apparatus (Stratagene, La Jolla, CA) through a 12% nondenaturing gel at 900 V for 12 hours at 4°C and exposed to Hyperfilm-MP (Amersham, Arlington Heights, IL) at −80°C. The autoradiogram was evaluated by densitometric scanning using the Ultrascan XL laser densitometer (Pharmacia LKB Biotech, Piscataway, NJ). Alternatively, PCR was performed under the same denaturation, annealing, and extension condition in the absence of radiolabeled dATP and the PCR products were electrophoresed in the SE 400 vertical slab gel unit (Hoefer Scientific Instruments, San Francisco, CA) for 7 hours at 150 V at room temperature through a 1.5-mm-thick 12% nondenaturing gel and stained with 1 mg/mL ethidium bromide for 30 minutes.

PCR products of five different sizes were detected, corresponding presumably to five different lengths of the dinucleotide repeat. Each of the five different bands was cloned into plasmid pCR II using the TA Cloning Kit (Invitrogen, San Diego, CA) and sequenced using the Sequenase Version 2.0 DNA Sequencing Kit (US Biochemical, Cleveland, OH) and the sequencing primer P476. Whenever two bands of different sizes were detected in genomic DNA, the subject was considered heterozygote for the $(AC)_n$ microsatellite size. The presence of a single band in genomic DNA was interpreted as homozygosity for the particular length of the AC repeat.

Comparison of the $(AC)_n$ microsatellite length in genomic DNA and cDNA. Total reticulocyte RNA was isolated from the heterozygous controls and patients by the ammonium chloride lysis technique,[19] reverse transcribed into cDNA using random hexamers and PCR amplified using the same conditions as for genomic DNA. For each heterozygous subject, PCR products from genomic DNA and cDNA were loaded into adjacent lanes, electrophoresed as described above, and the sizes of the PCR products obtained from genomic DNA and cDNA were compared.

RESULTS

Detection of combined deficiency of ankyrin and spectrin. Erythrocyte membrane proteins from 60 unrelated randomly chosen Czech patients with autosomal dominant HS were quantitated by SDS-PAGE electrophoresis followed by gel densitometry. Thirty-six (60%) unrelated HS patients were found to have an 8% to 35% decrease in the spectrin/band 3 ratio (65% to 92% of normal) and 15% to 37% decrease in ankyrin/band 3 ratio (63% to 85% of normal), indicating a combined deficiency of ankyrin and spectrin. These 36 unrelated HS patients were selected for further study.

Characterization of the AC repeat polymorphism. We PCR-amplified ankyrin genomic DNA from the 36 unrelated ankyrin-deficient HS subjects using primers P476 and P477, which flank the $(AC)_n$ microsatellite, and electrophoresed the PCR products in a 12% nondenaturing polyacrylamide gel. Using either autoradiography (Fig 1A) or ethidium bromide staining (Fig 1B), we observed five sizes of the PCR products, either individually or in combinations of two. The bands of the five different sizes were cloned and sequenced and found to contain 11, 12, 13, 14, and 15 AC repeats, respectively. We then quantitated the number of AC repeats

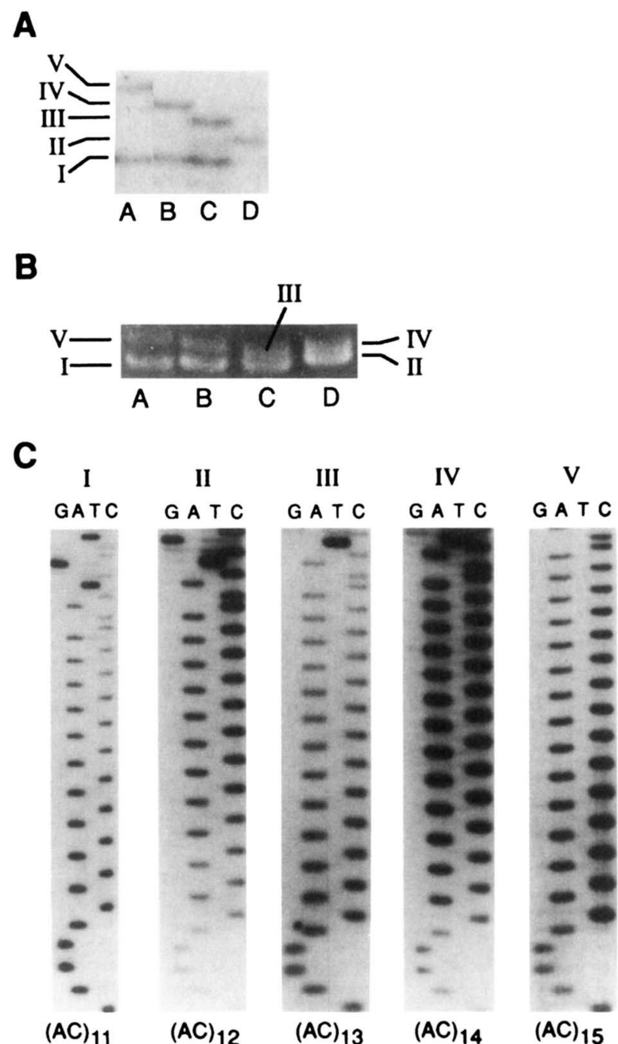

Fig 1. Characterization of the $(AC)_n$ repeat polymorphism in ankyrin genomic DNA. (A) The 3'-untranslated region of ankyrin genomic DNA containing the $(AC)_n$ microsatellite was PCR amplified using primers P476 and P477 in the presence of P^{32}-dATP and the reaction products were electrophoresed at 900 V for 12 hours at 4°C through a 12% nondenaturing gel that was subsequently exposed to Amersham Hyperfilm-MP. Products of five different sizes designated as bands I through V were detected. Each of the main bands was accompanied by a shadow band of ≈10% to 20% intensity of the main band, a phenomenon seen when typing dinucleotide and trinucleotide repeat polymorphisms.[20] (B) Alternatively, the same PCR amplification was performed in the absence of radiolabeled dATP and the reaction products were electrophoresed in an ethidium-bromide–stained 12% nondenaturing polyacrylamide gel. Again, five bands of differing sizes were detected. The shadow bands are not visible in this case, most likely because of the lower resolving capacity of this gel system. (C) Bands I through V were cloned and sequenced and found to contain 11, 12, 13, 14, and 15 AC repeats, respectively.

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Table 1. Distribution of the AC Repeat Length in the Studied Subjects

This Study			Data From Polymeropoulos et al[10]	
No. of AC Repeats	No. of Chromosomes (total of 36 individuals)	Frequency (%)	AC Repeat Length	Frequency (%)
11	28	0.39	n	0.54
12	3	0.04	n + 2	0.02
13	2	0.03	n + 4	0.04
14	38	0.53	n + 6	0.40
15	1	0.01	n + 8	ND

Abbreviation: ND, not detected.

in all the studied patients by comparing the size of their PCR products with the size of the sequenced bands. We found a bimodal distribution of the $(AC)_n$ length with 28 chromosomes (39%) containing 11 AC repeats, 3 chromosomes (4%) containing 12 AC repeats, 2 chromosomes (3%) containing 13 AC repeats, 38 chromosomes (53%) containing 14 AC repeats, and 1 chromosome with 15 AC repeats (Table 1). Genomic DNA from 21 of 36 patients (58%) was found to be heterozygous for the AC repeat length. Similar bimodal distribution of AC repeat sizes was detected by PCR amplification of genomic DNA from 15 unrelated healthy subjects, nine of whom (60%) were heterozygotes for the $(AC)_n$ length. Eight heterozygous controls had the combination of 11 and 14 AC repeats, while one (C5) was heterozygous for 11 and 13 AC repeats (Figs 2A and 3A).

Comparison of the AC repeat length in the genomic DNA and cDNA. Total reticulocyte RNA was isolated from the 21 patients and nine controls heterozygous for the AC repeat length, reverse transcribed using random hexamers and PCR amplified using the same conditions as for PCR amplification of the genomic DNA. The PCR products from both genomic DNA and cDNA from each patient were electrophoresed side-by-side in the 12% nondenaturing gels and the patterns of the genomic DNA and cDNA were compared. In all nine heterozygous control subjects, the heterozygosity for the ankyrin AC repeat-length polymorphism was readily demonstrable both in the ankyrin genomic DNA and cDNA (Figs 2A and 3A). In a striking contrast, a discrepancy between cDNA and genomic DNA (Figs 2B and 3B), ie, the presence of two ankyrin alleles of different AC length in genomic DNA, but only one of these alleles in the cDNA, was found in 7 of 21 (33%) heterozygous HS subjects with combined ankyrin and spectrin deficiency. The relative ankyrin content in these 7 patients ranged from 63% to 81% of normal. Because the biochemical phenotype of combined deficiency of spectrin and ankyrin was seen in 36 of 60 (60%) randomly chosen Czech patients with autosomal dominant HS, the absence of one ankyrin mRNA allele was detected in 33% of 60%, ie, in ≈20% of all studied HS patients.

Although the resolution of the different sizes of the PCR products was better in the autoradiograms, the main PCR bands were accompanied by shadow bands with the main shadow band migrating in the position corresponding to a PCR product shortened by two nucleotides. Presence of such bands is a common phenomenon seen during typing of dinucleotide and trinucleotide repeats[20-23] and is most likely caused by slipped mispairing during PCR.[20] Densitometric tracing of the autoradiograms showed that the most intense top shadow band was present in both genomic DNA and cDNA. Its intensity represented in all cases only 10% to 20% of the intensity of the main PCR product and was proportional to the amount of the PCR product loaded on the gel. Because of the weak intensity of the shadow bands relative to the main PCR products, the presence of the shadow bands does not interfere with the interpretation of the results.

The shadow bands are not visible in the smaller, ethidium-bromide–stained gels, most likely because of their lower resolving capacity. In this gel system, differences of four nucleotides are detectable (Fig. 1B, Fig 3A, control 1) and differences of six (Fig. 3A, controls 1-4 and 6-9, Fig. 3B, patients 1 through 6) or eight (Fig. 3B, patient 7) nucleotides are clearly visible. Because the difference between allele sizes in the seven heterozygous patients was six or eight nucleotides, the resolution obtained with this gel system was sufficient to show the absence of one ankyrin mRNA allele in all of them.

DISCUSSION

To examine whether a reduced ankyrin gene expression represents a common cause of HS, we took advantage of the $(AC)_n$ microsatellite–length polymorphism at the 3′ end of the ankyrin gene. We studied the distribution of the individual AC repeat sizes and found it to be bimodal, similar to that found in a different population,[10] suggesting that the

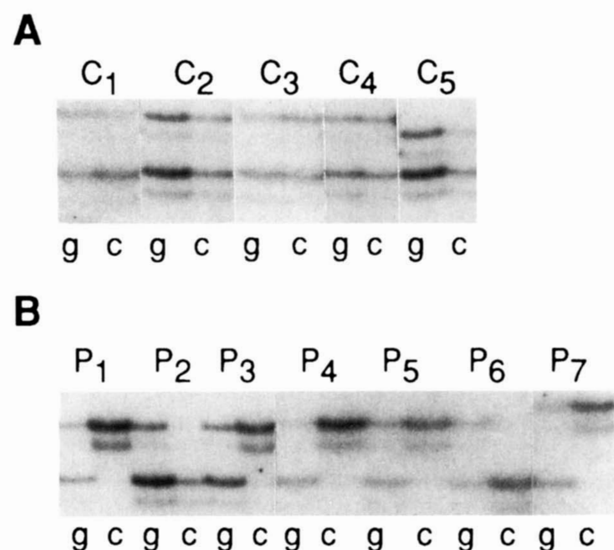

Fig 2. Comparison by autoradiography of the AC repeat polymorphism in genomic DNA and reticulocyte RNA in the $(AC)_n$ heterozygotes. (A) Simultaneous PCR amplification of genomic DNA (g) and cDNA (c) in the presence of P^{32}-dATP in the heterozygous control subjects did not show any differences in the pattern of the PCR products (results for five control subjects are shown). (B) The same procedure in the ankyrin-deficient HS patients showed two main bands in amplified genomic DNA and a single main band in amplified cDNA in 7 of 21 heterozygous subjects.

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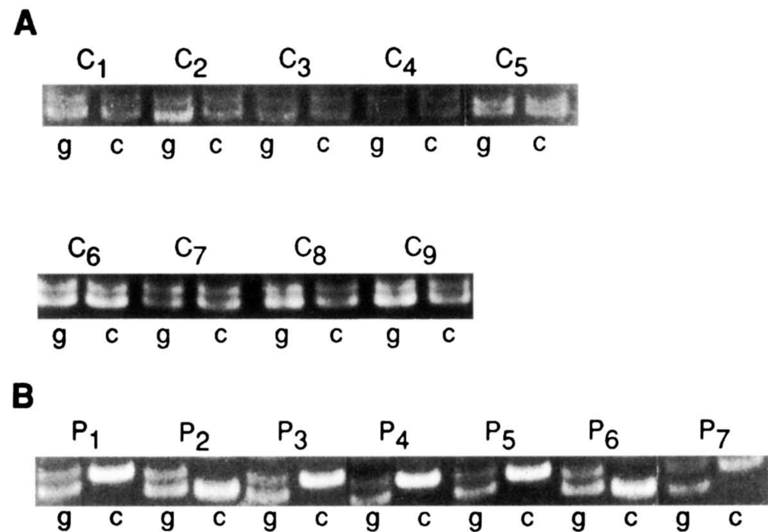

Fig 3. Comparison of the AC repeat polymorphism in the $(AC)_n$ heterozygotes in genomic DNA and reticulocyte RNA in ethidium-bromide–stained nondenaturing polyacrylamide gels. (A) Simultaneous PCR amplification of genomic DNA (g) and cDNA (c) in the nine heterozygous control subjects showed identical AC repeat patterns in the erythroid ankyrin genomic DNA and mRNA. (B) The same procedure in the twenty-one ankyrin-deficient heterozygous HS patients showed a doublet in genomic DNA and a singlet in cDNA in the same seven subjects as in Fig 2B, again suggesting the absence of one ankyrin mRNA allele.

variability of the ankyrin AC repeat length represents an ancient polymorphism. The predominant lengths of the AC repeat were 11 and 14 dinucleotides. In addition to the four sizes reported in Polymeropoulos et al,[10] we detected a repeat containing 15 AC dinucleotides in one patient. We found heterozygosity for the AC repeat polymorphism in genomic DNA from 21 of 36 (58%) patients, a finding which is in good agreement with the predicted heterozygosity of 56% based on the individual AC repeat-length frequencies. In 7 of 21 (33%) unrelated HS subjects heterozygous for the AC repeat length in the genomic DNA, only one of the two AC repeat sizes was detected in reticulocyte cDNA, indicating an absence in the reticulocyte mRNA of the product of one ankyrin allele.

Because of the expected differences in the resolution and artifacts observed in each of the two detection systems, ie, autoradiography of the sequencing-size gels and ethidium-bromide staining of small polyacrylamide gels, we chose to show electrophoresis of the PCR products in both gel systems. Evaluation of either gel system leads to identical conclusions. At the same time, each system has its drawbacks. In the case of the smaller size gels, the separation of bands becomes sufficient only for the difference in size of six or more nucleotides. Figure 1B shows the best resolution achieved in this gel system, which is not adequate for the two-nucleotide difference and marginal for the difference of four nucleotides. However, most heterozygotes carry the 11 and 14 AC microsatellites and the ankyrin alleles can be readily resolved (Figs 1B, 3A, 3B).

Band resolution is superior in the sequencing-size autoradiograms. However, PCR artifacts become visible, such as the "one repeat contraction product" commonly produced during amplification of dinucleotide and trinucleotide repeats. Existence of such products has been repeatedly described.[20-23] These "extra bands," "shadows," or "background constant bands"[24] are seen in practically all reports measuring the number of dinucleotide and trinucleotide repeats. The presence of an additional band shorter by two nucleotides than the main PCR product does not compromise differentiation between the two ankyrin alleles. The extra band is constant in all samples, both in genomic DNA and cDNA, and its intensity depends on the amount of PCR product loaded on the gel.

Although the presence of a detectable PCR product from only one ankyrin mRNA allele was shown using both gel systems (Figs 2B and 3B), being particularly apparent in the ethidium-bromide–stained gels (Fig 3B), the ratio of intensity of the individual PCR products fluctuated somewhat (Fig 2A and 3A), especially in the autoradiograms (Fig 2A). We conclude that the PCR-based approach, used in this study, is suitable for showing an all-or-nothing phenomenon, ie, an absence of detectable amounts of one PCR-amplified ankyrin mRNA allele, whereas other approaches, eg, RNase protection assay,[25] should be used for relative quantitation of mRNAs corresponding to the two ankyrin alleles.

We postulate that the absence of one ankyrin allele in reticulocyte mRNA is caused either by reduced transcription of the ankyrin gene or by instability of the mutant mRNA. Because this abnormality was found in approximately $\frac{1}{3}$ of all cases of HS with combined ankyrin and spectrin deficiency, and because this biochemical phenotype represents ≈60% of all HS cases, we suggest that a reduced expression of one ankyrin allele underlies ankyrin deficiency and, consequently, spectrin deficiency in ≈20% of all HS cases. Our conclusions are based on a presumption that both ankyrin alleles were present in all 36 studied patients, ie, in the total of 72 chromosomes. A de novo deletion of erythrocyte ankyrin gene on chromosome 8 was previously described in two unrelated children with severe HS.[6] Therefore, we cannot exclude that some of the HS subjects, whose genomic DNA appeared to be homozygous for the AC repeat length, were lacking one of the two ankyrin alleles. This could further increase the percentage of HS subjects in whom a reduced ankyrin gene expression underlies the HS phenotype.

In a recent preliminary report, screening of the ankyrin gene in 18 German HS patients for single-strand conformational polymorphisms (SSCPs) showed three frameshifts and one nonsense mutation interspersed throughout the coding

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region in four (22%) patients.[8] Adjustment of the observed 22% frequency of mutations for the estimated 70% yield of SSCP screening yields a 31% predicted frequency of frameshifts and nonsense mutations in the ankyrin gene in German HS patients with ankyrin deficiency. It has been well documented that nonsense mutations in messenger RNAs, with the exception of the 3′ ends of the coding regions, cause a reduction in steady-state mRNA levels by promoting rapid degradation of mutant mRNA molecules.[9] Consequently, the predicted frequency of ankyrin frameshifts and nonsense mutation in the German population appears to be nearly identical to the observed frequency of the absence of one ankyrin allele from the reticulocyte mRNA in the neighboring Czech population.

In conclusion, using the technique of simultaneous screening of genomic DNA and cDNA for exonic AC repeat polymorphism, we showed that the absence in reticulocytes of mRNA corresponding to one of the two ankyrin alleles is observed in ≈20% of randomly selected patients with autosomal dominant HS. We suggest that the underlying mechanism involves either abnormal transcription of one of the two ankyrin alleles, or abnormal processing or decreased stability of the mutant ankyrin mRNA. Because numerous polymorphic dinucleotide and trinucleotide repeats are found in the human genome, the same technique of simultaneous screening of the microsatellite length in genomic DNA and mRNA can be applied for the study of expression of a number of other genes.

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