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Characterization of a Spontaneous Mutation to a β-Thalassemia Allele

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SUMMARY

We have studied a nuclear family containing a single child with severe β-thalassemia intermedia, a Greek-Cypriot mother with hematological findings of β-thalassemia trait, and a Polish father who is hematologically normal. Since both the child and her father were heterozygous for a DNA polymorphism within the β-globin gene, it was possible to clone and sequence the β-globin gene identical by descent from both the child and her father. A nonsense mutation in codon 121 (GAA→TAA) was found in the β-globin gene of the child, while the same gene from her father lacked this mutation and was normal. This mutation has not been previously observed among over 200 β-thalassemia genes characterized in Caucasians. Since the mutation eliminates an *Eco*RI site in the β-globin gene, we could show that the mutation is not present in genomic DNA of the father. To rule out germinal mosaicism, sperm DNA of the father was also digested with *Eco*RI, and the mutant *Eco*RI fragment was not observed under conditions that would detect the mutation if it were present in at least 2% of sperm cells. Routine HLA and blood group testing supported stated paternity. In addition, studies with 17 DNA probes that detect multi-

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ple allele polymorphisms increased the probability of stated paternity to at least 10^8:1. These data provide evidence that the G→T change in codon 121 of the β-globin gene in the child is the result of a spontaneous mutation that occurred during spermatogenesis in a paternal germ cell.

INTRODUCTION

Spontaneous mutation has been well documented for X-linked disorders, including Duchenne muscular dystrophy [1, 2], and many autosomal dominant disorders, such as achondroplasia [3]. However, few new recessive mutations have been characterized. Evidence has been presented for new mutations to β-thalassemia trait in two families [4, 5], but these instances, while probably bona fide examples of the phenomenon, have not yet been characterized at the molecular level.

β-Thalassemia major is a severe inherited anemia in which both β-globin alleles are altered by mutation to reduce their expression [6]. The disorder is heterogeneous, and a large number of different mutations to β-thalassemia have been characterized with each ethnic group carrying its own particular set of mutations [7, 8]. The condition is prevalent in areas of the world in which malaria is endemic, that is, the Mediterranean region, parts of India, China, Southeast Asia, and Africa.

Because of the geographical distribution of β-thalassemia, it was of great interest to study the condition in a family in which one parent, the father, was of Polish extraction. In addition, he was hematologically free of stigmata of β-thalassemia trait. His child, however, was affected with severe β-thalassemia intermedia and required transfusions on a regular basis. Here, we present evidence indicating that a spontaneous mutation to β-thalassemia, a single nucleotide substitution producing a previously undescribed nonsense mutation in the β-globin gene, has occurred in a paternal germ cell.

MATERIALS AND METHODS

Analysis of DNA Polymorphisms

High molecular weight DNA was prepared as described [9]. Restriction site polymorphisms in the β-globin gene cluster were analyzed [10]. The particular haplotype of the β-globin gene cluster was determined by analysis of these sites in various members of the family. The DNA fragments used as probes for these analyses have been described [10].

Gene Cloning and DNA Sequencing

The β-thalassemia genes were cloned in bacteriophage λ Charon 28 as 7.5-kilobase (kb) *Hin*dIII fragments and then subcloned as 3.7-kb *Bgl*II-*Pst*I fragments in the expression plasmid πSVplac [11]. DNA sequence analysis was by the method of Maxam and Gilbert [12].

Paternity Testing by RFLP Analysis

Probes for multiple allele polymorphisms were isolated at Collaborative Research, from the genomic DNA library of Lawn et al. [13]. They are randomly selected, single-copy sequences that were screened in Southern blot hybridizations for restriction fragment length polymorphism in Caucasians from the Boston area. The polymorphism information content (PIC) values were calculated as described by Botstein et al. [14] from allele frequencies measured in a Utah population. Analysis of genomic restriction fragments in Southern blot hybridizations was performed by the methods of Barker et al. [15].

RESULTS

The nuclear family is shown in figure 1. The child had β^0-thalassemia with 97% HbF and 3% HbA_2. She had all the clinical and laboratory features of the disease. The mother, a Greek Cypriot, had classic β-thalassemia trait with a low MCV and 5.6% HbA_2. The father, however, had a normal peripheral blood count, red cell morphology (MCV = 90 fl.), and hemoglobin electrophoresis. The father was 35 years old and the mother was 34 years old at the time of conception. Since the mother denied incorrect paternity, and the couple wished further children, the family was studied thoroughly. DNA haplotypes in the β-globin gene cluster were determined for each of the family members (see fig. 1). The haplotype of both thalassemia genes in the daughter, the normal and thalassemia genes in the mother, and the normal and "suspect thalassemia" genes in the father were determined. It was possible to distinguish the two alleles at the β-globin locus in both daughter and father by the presence or absence of the polymorphic *Ava*II site in the β-globin gene.

Thus, the β-globin genes of both daughter and father were cloned in Charon 28A and the *Ava*II (−) allele of both members was chosen for DNA sequence analysis. This analysis demonstrated that these β-globin genes from both father and daughter contained the DNA polymorphisms of framework 3 β genes (codon 2, IVS-2 nt 16, IVS-2 nt 74, IVS-2 nt 81, IVS-2 nt 666) [16]. However, these β-globin genes differed in that the first nucleotide of codon 121 was G in the

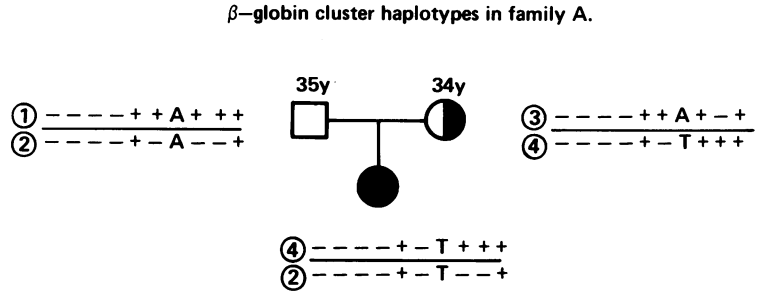

FIG. 1.—β-Globin cluster haplotypes in family A. Haplotypes were constructed for the two β-globin clusters in mother, father, and affected child using the following polymorphic sites *from left to right: Hin*dIII-$^{G}\gamma$, *Hin*dIII-$^{A}\gamma$, *Hin*cII-$\psi\beta_1$, *Hin*cII-3′ $\psi\beta_1$, *Hin*fI-5′ β, *Rsa*I-5′ β, *Ava*II in β, *Hin*dIII-3′ β, and *Bam*HI-3′ β. By this analysis, the child received β-globin cluster [4] from her mother and [2] from her father. The β-globin gene from [2] was cloned in both child and father by use of heterozygosity at the *Ava*II site in the β gene.

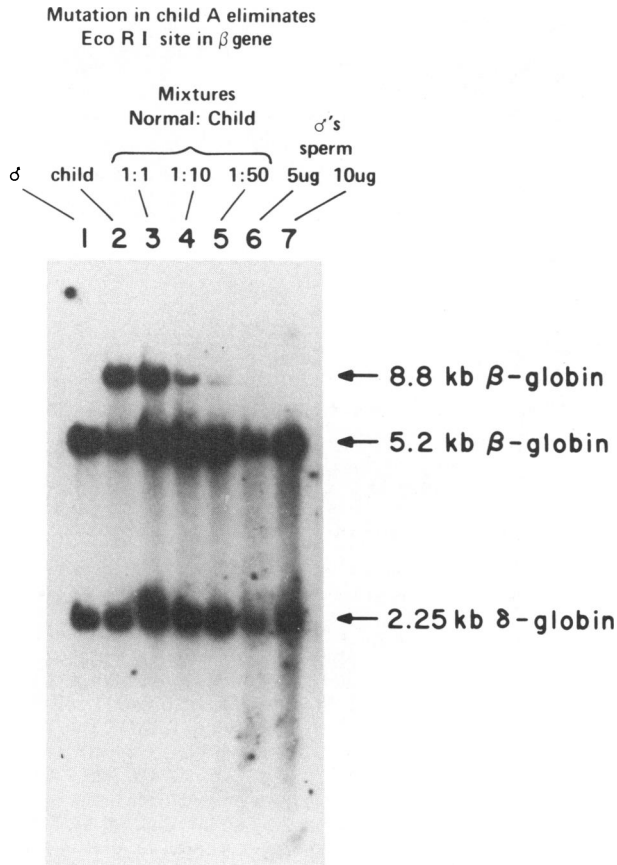

FIG. 2.—Mutation in child A eliminates an *Eco*RI site in the β-globin gene. Digestion of genomic DNA of father and child (*lanes 1* and *2*) reveals absence of the *Eco*RI site in one β gene of the child, while both β genes of the father are normal and contain the site. *Lanes 3–5* are digestions of various mixtures of normal and child's DNA with *Eco*RI. *Lanes 6* and *7* are digestions of 5 μg and 10 μg of DNA derived from paternal sperm with *Eco*RI. These latter digests show no trace of the abnormal 8.8-kb *Eco*RI fragment, while this fragment is easily seen in a 50:1 mixture of normal DNA with DNA of the child.

father's gene and T in the daughter's gene. Thus, codon 121 was GAA (normal) in the father but was TAA (nonsense codon) in the daughter. Fortuitously, the mutation in the daughter's mutant gene also eliminates the *Eco*RI site at codons 120 and 121 (fig. 2). The presence of this mutation in the daughter and its absence in the father was documented by restriction analysis (fig 2). These data suggested that a spontaneous mutation had arisen in the father's germ cells.

Two other pieces of evidence were obtained to support this conclusion. First, considerable effort was made to verify the stated paternity. Conventional HLA and blood group studies could not exclude stated paternity at the level of 98.9%. Studies with 17 DNA probes for polymorphic loci were carried out on DNA samples from the father, mother, and affected child (fig. 3). The allele frequencies for each polymorphism, expressed as the polymorphism informa-

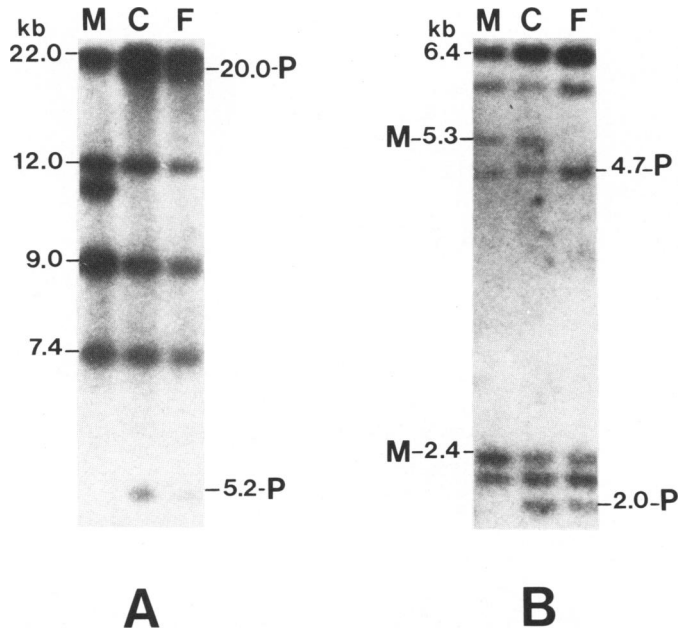

FIG. 3.—Confirmation of paternity by genotypic analysis with DNA probes. DNA samples from the mother (*M*), affected child (*C*), and father (*F*) were digested with restriction enzymes and tested with each of the probes listed in table 1. Studies with two of the 17 polymorphic probes used are shown as examples. *A*, *Bgl*II-digested DNAs hybridized to LAM4-1214. The child's hybridization pattern includes fragments at 20 kb and 5.2 kb that are absent in the mother and must have been inherited from her father. Fragments at 22 kb, 12 kb, 9.0 kb, and 7.4 kb could have been inherited from either parent. *B*, *Pst*I-digested samples probed with LAM4-159. With this probe-enzyme combination, DNA fragments at 5.3 kb and 4.7 kb are allelic as are DNA fragments at 2.4 kb and 2.0 kb. All other fragments are not polymorphic. Therefore, the child inherited the 4.7-kb and 2.0-kb fragments from her father and the 5.3-kb and 2.4-kb fragments from her mother. *M* and *P* indicate known maternal and paternal fragments, respectively.

tion content or PIC [14], were measured in a Utah population. PIC values for these 17 probes ranged from 0.58 to 0.95 [17] (table 1). In every case, the genotypic patterns in Southern blot hybridizations were consistent with the expected paternity. A priori, we would expect from the PIC values of the probes that the probability of nonpaternity (i.e., the probability that the same alleles would be contributed by a random person) would be 1.3×10^{-12}. (For a highly polymorphic marker, the probability of two individuals contributing the same allele is very close to 1 − PIC.) In practice, the paternal alleles of many tested loci cannot be unambiguously determined from the band patterns of these three family members, and the contribution of each probe to establishing paternity is less than theoretically predicted. Nevertheless, when we add the frequencies of all alleles of each locus that could potentially represent the child's paternal allele (table 1), we calculate that the probability of nonpaternity is 2×10^{-9}.

Second, we attempted to rule out the presence of two populations of sperm in the father, one with the mutation and the other lacking it. DNA was isolated

TABLE 1

POLYMORPHIC DNA PROBES FOR GENOTYPIC ANALYSIS OF THE AFFECTED CHILD AND HER PARENTS

Probe	Enzyme	PIC	Frequency of potential paternal alleles
LAM4-45	*Msp*I	.60	.34
LAM4-159	*Pst*I	.90	.06
LAM4-355	*Bgl*II	.90	.04
LAM4-368	*Hin*dIII	.58	.23
LAM4-281	*Msp*I	.70	.20
LAM4-1020	*Taq*I	.80	.37
LAM4-1214	*Bgl*II	.94	.17
RL4-265	*Bgl*II	.75	.25
RL4-392	*Bgl*II	.65	.43
LAM4-123	*Rsa*I	.81	.69
LAM4-171	*Msp*I	.70	.11
LAM4-427	*Rsa*I	.95	.69
LAM4-605	*Msp*I	.70	.53
LAM4-966	*Rsa*I	.88	.65
LAM4-1065	*Rsa*I	.75	.71
LAM4-1209	*Taq*I	.70	.93
LAM4-962	*Msp*I	.70	.66

NOTE: The polymorphism information content (PIC) is the probability that the alleles in a family are informative for inheritance [14]. For each of the polymorphic loci, those alleles that could account for the restriction fragment patterns seen in the affected child (given the mother's genotype) were classified as potential paternal alleles. The sum of the frequencies of all the potential paternal alleles is then the probability that an allele from a random individual would give the same pattern as the one observed, and the probability that alleles at all 17 loci would be consistent with this pattern is the product of these values, 2×10^{-9}.

from the father's sperm and studied for the presence of the abnormal *Eco*RI fragment that marks the nonsense codon 121 mutation (fig. 2). We did not observe the altered fragment produced by elimination of the *Eco*RI site in the β-globin gene. Analysis of mixtures of DNA from a control individual and the affected child demonstrated that such a fragment could be detected if it were present in 2% of sperm cells. Thus, significant germinal mosaicism of the mutation was ruled out.

DISCUSSION

There have been two previous reports of spontaneous mutation to a β-thalassemia allele [4, 5]. In each of these reports, hematologically normal parents gave birth to a child with β-thalassemia trait. Incorrect paternity was ruled out at the 99% level in each, but no molecular characterization was carried out because it was not available at the time. In neither report was it possible to state in which parent the purported spontaneous mutation had occurred.

This report goes beyond these two previous ones in that (1) the parent in whom the suspected spontaneous mutation occurred is known, (2) efforts to rule out incorrect paternity were very extensive, (3) the molecular defect that occurred in a paternal germ cell was elucidated, and (4) significant paternal germinal mosaicism was excluded.

Although the question of nonpaternity is always difficult to resolve in a case such as this, considerable data favor the stated paternity. First, the mutation contributed by the father has not been previously observed among over 200 Mediterranean and Caucasian β-thalassemia genes studied by us [18]. Second, the mother denies any possibility of nonpaternity. Third, the analysis of multiple allele polymorphisms increased the probability of stated paternity to at least 10^8:1.

It should also be noted that the father's age at the time of conception (35 years) is well above the mean for paternal age. Advanced paternal age has been shown to increase the risk of spontaneous mutation, particularly to new dominant diseases [19, 20].

Although spontaneous mutation has been well documented in many dominant and X-linked disorders, the example presented here is one of the first spontaneous mutations to be well characterized at the nucelotide level. To our knowledge, only two other new mutations (a tandem duplication in the *HPRT* gene in a male with the Lesch-Nyhan syndrome and a nonsense mutation in a hemophiliac) have been similarly characterized [21, 22].

The GAA→TAA nonsense mutation at codon 121 of the β-globin gene increases the known number of different point mutations leading to β-thalassemia to 37. It is worth noting that all possible nucleotide substitutions of the first nucleotide, G, of codon 121 have now been observed. The G→A substitution produces the relatively frequent variant β-globin, $\beta^{O\ Arab}$, in blacks [23], while the G→C substitution leads to the common variant $\beta^{D\ Punjab}$ in Asian Indians [24].

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