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Identification of a Frameshift Mutation Responsible for the Silent Phenotype of Human Serum Cholinesterase, Gly 117 (GGT→GGAG)

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Summary

A frameshift mutation that causes a silent phenotype for human serum cholinesterase was identified in the DNA of seven individuals of two unrelated families. The mutation, identified using the polymerase chain reaction, causes a shift in the reading frame from Gly 117, where GGT (Gly)→GGAG (Gly+ 1 base) to a new stop codon created at position 129. This alteration is upstream of the active site (Ser 198), and, if any protein were made, it would represent only 22% of the mature enzyme found in normal serum. Results of analysis of the enzymatic activities in serum agreed with the genotypes inferred from the nucleotide sequence. Rocket immunoelectrophoresis using alpha-naphthyl acetate to detect enzymatic activity showed an absence of cross-reactive material, as expected. One additional individual with a silent phenotype did not show the same frameshift mutation. This was not unexpected, since there must be considerable molecular heterogeneity involved in causes for the silent cholinesterase phenotype. This is the first report of a molecular mechanism underlying the silent phenotype for serum cholinesterase. The analytical approach used was similar to the one we recently employed to identify the mutation that causes the atypical cholinesterase variant.

Introduction

The genetically determined deficiency of human serum cholinesterase, called the silent cholinesterase phenotype, is characterized essentially by a complete lack of enzymatic activity. Individuals homozygous for this trait develop prolonged apnea following the administration of the muscle-relaxant drug succinylcholine in standard doses (Lehmann and Ryan 1956). Ordinarily, this drug is extensively metabolized by serum cholinesterase, as it was reported by F. Bovet-Nitti in 1949 (see Liddell et al. 1962). The frequency of the silent gene is estimated to be .003 in the Caucasian population, and about 1:100,000 individuals should be homozygous for the silent allele (Hodgkin et al. 1965).

The presence of a silent gene was initially predicted in order to explain the apparent distortion in the segregation of usual and atypical phenotypes of cholinesterase in two families (Kalow and Staron 1957; Harris et al. 1960). A few years later, two individuals homozygous for the silent allele were found (Hart and Mitchell 1962; Liddell et al. 1962), and since then many additional cases of this phenotype have been reported. These individuals do not develop any pathological condition that can be attributed to the absence of the enzyme, except for the exaggerated response to succinylcholine. It was shown by Simpson and Kalow (1964) that the silent trait is allelic with the usual and atypical genotypes.

Although the frequency of the silent phenotype in Caucasian populations is extremely low, it is consider-

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ably higher in other populations, approaching 1% among Eskimo in western Alaska (Gutsche et al. 1967) and 2% in the population of Andhra Pradesh, India (Rao and Gopalam 1979). It was also observed in the Eskimo population that some of the homozygotes for the silent allele had a very low level of cholinesterase, (2%–10% of normal values), while others had absolutely no detectable activity in the assay, when benzoylcholine was used as a substrate (Gutsche et al. 1967). This observation was in agreement with the hypothesis of heterogeneity among silent phenotypes, a hypothesis proposed by other authors (Goedde et al. 1965; Goedde and Altland 1968).

Rubinstein et al. (1970) analyzed a collection of 25 sera from individuals classified as homozygous for the silent allele, by a combination of different techniques (serum activity with different substrates and inhibitors, immunological and chromatographic methods). They found that most of the sera with silent phenotype could be classified into two groups: (1) type I, in which there is no activity of serum cholinesterase and in which absence of cross-reactive material and the biochemical properties of a trace activity were similar to those of the residual red blood cell ghosts acetylcholinesterase, present both in normal sera and in sera with silent phenotype, and (2) type II, which showed about 2% of the activity of normal serum cholinesterase and the presence of cross-reactive protein. Scott (1970) purified trace enzyme from individuals with the silent type II allele and concluded that this enzyme was surely cholinesterase, even though it had a greater heat stability and a lesser degree of inhibition by excess of substrate, compared with the usual serum cholinesterase. One serum with silent phenotype was also found with intermediate properties; it could not be classified as either type I or type II (Rubinstein et al. 1970). The two types of cholinesterase deficiency, called E_1^s (type I) and E_1^t (type II), were believed to be allelic (Scott 1973).

Scott and Wright (1976) described a third variant, called E_1^r, in Alaskan Eskimos; it seemed to be allelic to E_1^s and E_1^t, which had been previously described in this population. This variant had less than 10% of the normal activity with benzoylcholine, it had electrophoretic mobility slightly greater than that of the usual enzyme, and it gave an unusually strong reaction with alpha-naphthyl acetate in the zymogram. There are further reports of silent phenotypes presumably caused by other alleles (Das 1973; Lubin et al. 1973; Evans and Magill 1974; Arnason et al. 1975).

Other variants, which presented activity in the assay with benzoylcholine but which had no activity with other substrates—such as acetylcholine (Simpson 1968) or succinylcholine (Agarwal et al. 1976)—have been described. The latter is caused by the allele called E_1^{su} (Goedde and Agarwal 1979), and homozygous individuals develop apnea after administration of succinylcholine. Although no family pedigree was given to support the hypothesis, these results suggest that there exist other cholinesterase variants that can be identified by using substrates other than benzoylcholine. Prody et al. (1989) reported, in a family from Israel, an association between defective ("silent") serum cholinesterase and in vivo amplification of the gene for this enzyme, an association presumably caused by prolonged exposure to organophosphorus insecticides.

We recently described the use of the techniques of amplification by polymerase chain reaction (PCR) and sequencing of DNA to characterize the mutation responsible for the atypical serum cholinesterase (McGuire et al. 1989). In the present report, we describe our results when this same approach is used to characterize, for the first time, a molecular mechanism that causes one kind of silent phenotype of human serum cholinesterase.

Material and Methods

Blood Samples

Serum and white blood cell (WBC) DNA from both (1) patients reported to have prolonged muscle relaxation following the administration of succinylcholine and (2) their family members were analyzed. Extraction of DNA from WBC was performed according to the method described by Maniatis et al. (1982), with slight modifications.

Determination of the Cholinesterase Phenotype

Characterization of the serum cholinesterase phenotype was made by measuring activity by means of benzoylcholine (Kalow and Lindsay 1955) and by determining the dibucaine (Kalow and Genest 1957) and fluoride (Harris and Whittaker 1961) numbers.

Amplification Using PCR and Sequencing

Genomic DNA was amplified with *Taq* polymerase (Saiki et al. 1988) by using 1–2 μg of the WBC DNA digested with *Hin*dIII, in the same amplification mixture as described in our previous paper (McGuire et al. 1989). A typical amplification was performed with 45 cycles of denaturation (94°C for 1.5 min), annealing (50°C for 1.5 min), and extension (72°C for 3 min), using 5 units of *Taq* polymerase (Cetus).

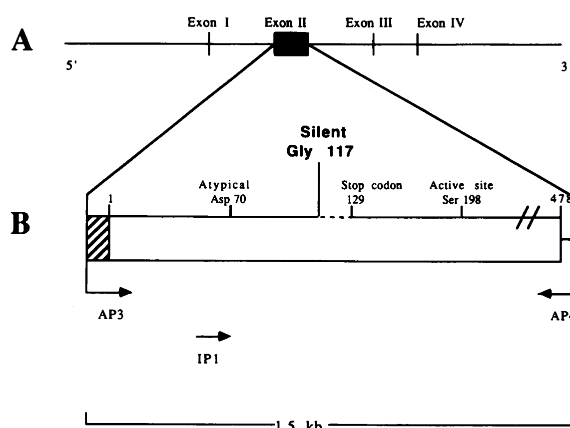

Figure 1 Schematic representation of the gene of serum cholinesterase. *A*, Entire gene (size estimated to be about 73 kb). *B*, Magnification of the region amplified by amplification primers AP3 and AP4, including all of exon II, showing the internal primer used for sequencing (IP1), the site of the mutation that causes the silent phenotype on Gly 117, the atypical site at Asp 70, the area of the frameshift (interrupted line), and the signal peptide (cross-hatched). Number 1 is the first amino acid at the N-terminal of the mature protein.

DNA of individuals from families 1 and 2 (fig. 2) was analyzed, using amplification with specific primers—35-mer primers AP3 and AP4 (fig. 1)—which gave a 1.5-kb fragment of exon II. Occasionally other primers were used, to obtain smaller fragments. The region of the frameshift was sequenced with an internal 20-mer primer, IP1, end-labeled with T4 polynucleotide kinase and gamma ^{32}P-ATP (Maniatis et al. 1982), and purified on 20% polyacrylamide gel. DNA was sequenced by the method of Sanger et al. (1977).

Other regions of exon II and exons III (167 bp) and IV (122 bp), from the silent homozygote I-2 from family 1 (fig. 2), were also analyzed by amplification and sequencing with primers that included the entire coding region (McGuire et al. 1989).

Immunoelectrophoresis

Rocket immunoelectrophoresis was performed in 1% agarose gels (Laurell 1966), using rabbit antibody against human cholinesterase (Accurate). The gel and bridge buffer contained 0.025 M and 0.050 M sodium barbital pH 8.6, respectively. Electrophoresis was performed at 10 V/cm for 3 h. Gels were rinsed with the staining buffer to remove nonprecipitated materials and then were stained with alpha-naphthyl acetate and Fast Blue RR in 0.2 M phosphate buffer pH 7.4 (Stern and Lewis 1962). The serum with silent phenotype, used for comparison, previously had been characterized as not having cross-reactive material, by using Ouchterlony double-diffusion technique. Purified human serum cholinesterase used as a standard in this experiment was prepared in our laboratory (Lockridge and La Du 1978).

Genomic Blots

WBC genomic DNA (8–10 μg) was digested with *Eco*RI or *Hin*dIII, submitted to electrophoresis in 1% agarose gel, transferred to a nylon membrane (GeneScreen), and hybridized with probes labeled with gamma ^{32}P-dCTP, containing exons I–IV of the human cholinesterase gene (Arpagaus et al. 1990). Hybridization was at 60°C in 6 × SSC, 0.25% nonfat dry milk. Nylon membranes were washed in 2 × SSC, 0.1% SDS at 60°C, 1 h for each, with three buffer changes.

Results

Activity of Serum Cholinesterase

Patient Mi.M. (I-3, family 1; fig. 2) was identified as having an exaggerated response to succinylcholine; the assay for cholinesterase activity in her serum showed no detectable activity. Serum from her husband or from her parents was not available, but it was known that her parents are first-degree cousins. Her two daughters (II-1 and II-2), obligatory heterozygotes, showed intermediate values for activity and had normal dibucaine and fluoride numbers, compatible with the US phenotype. One brother of the propositus (I-2) also showed no cholinesterase activity in serum; another brother (I-1) had normal activity. In family 2, the father (I-1) had values for activity in serum that were compatible with the US phenotype; the mother was a heterozygous UA, and both children (II-1 and II-2) were AS.

Analysis of DNA

Sequencing of the WBC DNA of the propositus (I-3) in family 1 showed, in the codon corresponding to Gly 117, the mutation GG<u>T</u>→GG<u>AG</u>, i.e., both a point mutation in the third base of the codon and an extra base (fig. 3). The extra base causes a frameshift, which changes the reading frame (+1) from Gly 117 to codon 129, where a stop codon is created (fig. 4). The frameshift and the stop codon are both upstream of the active site, Ser 198 (McTiernan et al. 1987). Only 128 amino acids could be translated, so if the corresponding polypeptide were produced in the individual with the silent genotype, it would be only 22% of the length of the serum enzyme.

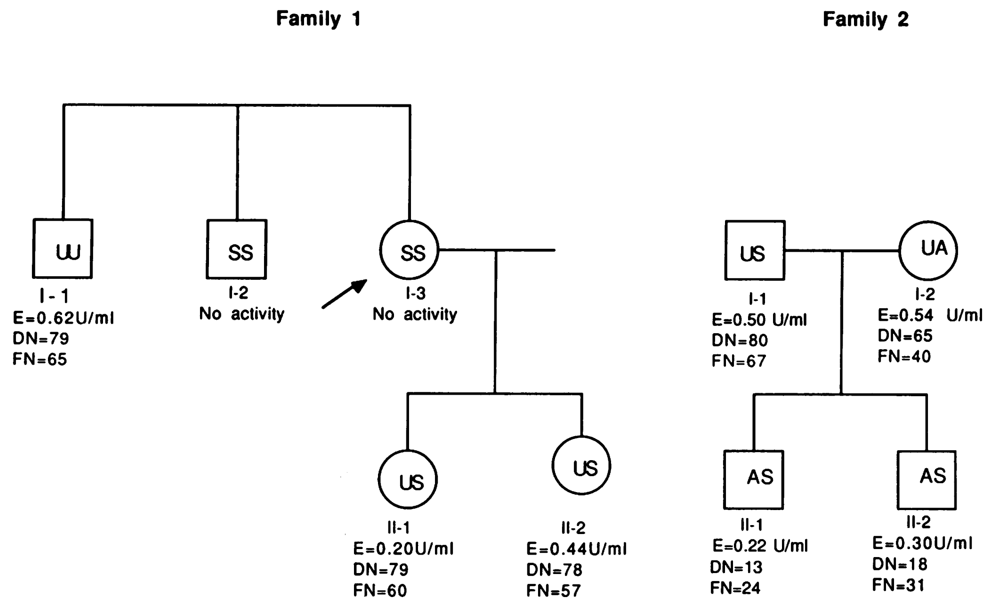

Figure 2 Segregation of the silent (S), usual (U), and atypical (A) alleles of serum cholinesterase in two pedigrees. The arrow indicates the propositus in family 1. E = enzymatic activity in serum (normal range, 0.65–1.01 U/ml); DN = dibucaine number (range for UU and US, 77–83; range for UA, 48–69; range for AA and AS, 8–28); FN = fluoride number (range for UU and US, 56–68; range for UA, 44–54; range for AA and AS, 10–28).

The inheritance of this mutation was confirmed by analysis of the DNA of other members of family 1 (fig. 2). The pattern observed was also compatible with the serum phenotype: individual I-1 (UU) showed the usual codon at this position, GGT (Gly), and the heterozygous children II-1 and II-2 showed the pattern of double bands characteristic of heterozygotes with a frameshift mutation (fig. 3).

This same frameshift mutation was observed in the second family (fig. 2). This family had the atypical al-

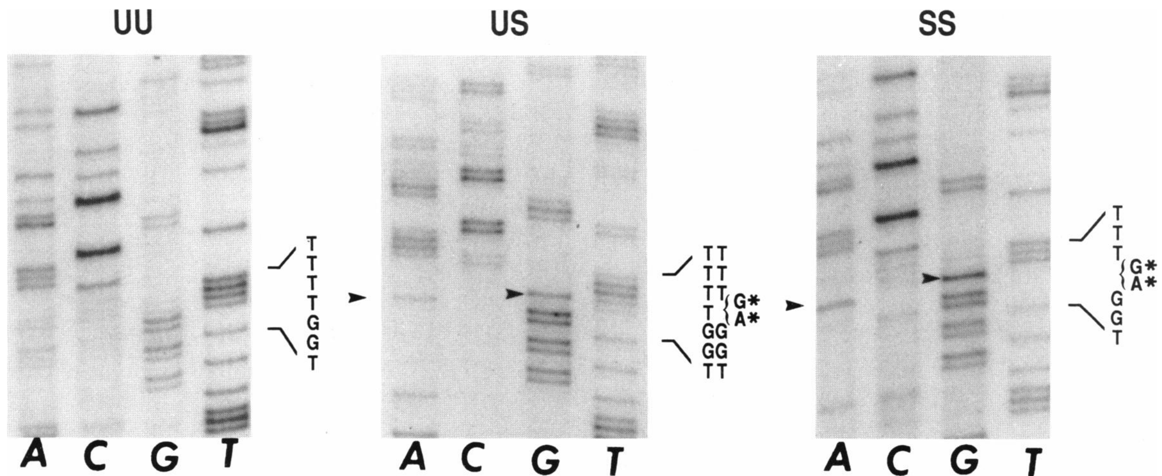

Figure 3 Autoradiograph of sequencing gels (direct sequencing of DNA), showing both alleles in individuals from family 1: the usual homozygote (I–1), the silent individual (I–2), and the heterozygote (II–2) in the region of the frameshift, Gly 117, GGT→GGAG (arrows). Asterisks indicate the bases that differ from the usual sequence. The heterozygote has the characteristic pattern of duplicate bands for all base positions after the site of the frameshift.

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Gly 117: GGT->GGAG  (GGA, GGG, GGT=Gly).

   115      117             120               125            129
U: Gly  Gly  Gly   Phe  Gln  Thr  Gly  Thr  Ser  Ser  Leu  His  Val Tyr Asp ...
   GGT GGT GGT  TTT  CAA ACT GGA ACA TCA TCT TTA CAT GTT TAT GAT...
   |||  |||  |||
S: GGT  GGT  GGA  GTT  TCA  AAC TGG  AAC  ATC ATC TTT  ACA  TGT TTA  TGA***
   Gly  Gly  Gly   Val  Ser  Asn  Trp  Asn  Ile  Ile  Phe Thr  Cys  Leu  STOP

     |-------------|
   Tandem   repeat
                 |----------------------Reading  frame  +1----------------|
```

Figure 4 Sequence of nucleotides and corresponding amino acids (115–129) in exon II of the gene for serum cholinesterase. U = usual genotype; S = silent genotype with both a frameshift on Gly 117 (GG<u>T</u>→GG<u>AG</u>) and a stop codon on Asp 129. Characters in boldface and underlined denote the changes in the sequence that are caused by the mutations; the tandem repeat on Gly 115–117 is shown in italics.

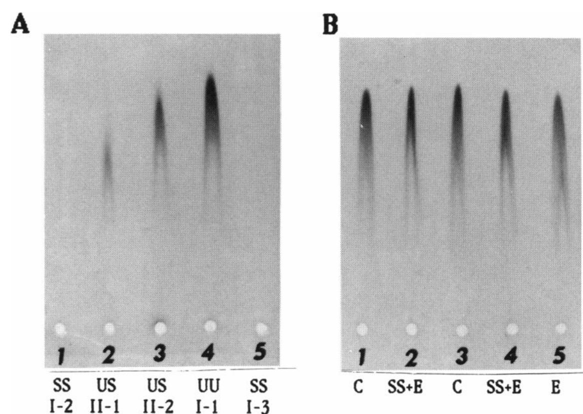

Figure 5 Rocket immunoelectrophoresis for serum cholinesterase in individuals from family 1. *A*, Serum (3 μl) of the homozygotes for the usual (lane 4) and silent (lanes 1 and 5) alleles and for heterozygotes (lanes 2 and 3). *B*, Samples (1.6 μU) of purified cholinesterase (by itself on lane 5) mixed with 5 μl of serum from the silent homozygote (lanes 2 and 4) and with serum from another individual with silent phenotype with no cross-reactive material (lanes 1 and 3). Gels were stained for activity by using alpha-naphthyl acetate and Fast Blue RR.

lele, in addition to the silent one, and sequencing of position 70 confirmed the presence of GGT (Gly) instead of GAT (Asp), (McGuire et al. 1989). From analysis of the pedigree it also can be inferred that the frameshift mutation is not linked to the atypical allele but that it is, instead, carried on the other chromosome.

The whole coding region, exons II–IV (1,722 bp), of one of the homozygotes for the silent allele on family 1 (I-2) was sequenced, and no abnormalities other than the frameshift were found. Another individual, unrelated to families 1 and 2 and previously identified in our laboratory as having the silent phenotype, was investigated at the site of the above-mentioned frameshift, but she was shown not to have the mutation.

Analysis for Cross-reactive Material

Rocket immunoelectrophoresis was performed on the serum of most members of family 1, to look for the presence of cross-reactive material, by using activity with alpha-naphthyl acetate to identify serum cholinesterase (fig. 5*A*) The homozygotes for the silent allele (I-2 and I-3) showed no peaks, as expected (lanes 1 and 5). The heterozygous children (II-1 and II-2) had peaks shorter and less intense than that of the usual type (I-1) in the family, suggesting either that no cross-reactive material was produced by the silent allele or, if it is produced, that it was below the level of detection by the methods commonly utilized. The two heterozygotes also had different amounts of cross-reactive material, a result in agreement with heterozygous II-1 having about half of the activity of heterozygote II-2. Comparison of individual I-1 (UU) with other usual individuals showed that his values for serum cholinesterase (activity and amount of cross-reactive material) are lower than the average for the usual type (data not shown).

Experiment A was informative for the heterozygotes but was not fully informative for the silent phenotypes, because the lack of activity in this phenotype prevented visualization of cross-reactive material, if it were present. For this reason, a known amount of purified cholinesterase was added to two sera with silent phenotypes, one under investigation (I-2 in fig. 2) and one previously identified as having no cross-reactive material. The peaks obtained for both of these sera were very similar (lanes 1–4), confirming the absence of cross-reactive material in the silent genotype with the frameshift mutation. The peaks were slightly higher than that obtained for the enzyme by itself, and this was probably due to differences in the amount of other proteins in the samples; it was not consistently found in other experiments.

Genomic Blots

The pattern of bands observed in the genomic blots (WBC DNA) was the same in individuals with phenotypes UU, US, and SS (family 1). The DNA, digested with *Eco*RI (fig. 6) or *Hin*dIII (1.3, 4.4, 5, and 10.5 kb; data not shown), yielded the same bands previously reported (Arpagaus et al. 1990). These results excluded the possibility of major alterations in the structure of the gene associated with the silent phenotype, as observed by Prody et al. (1989).

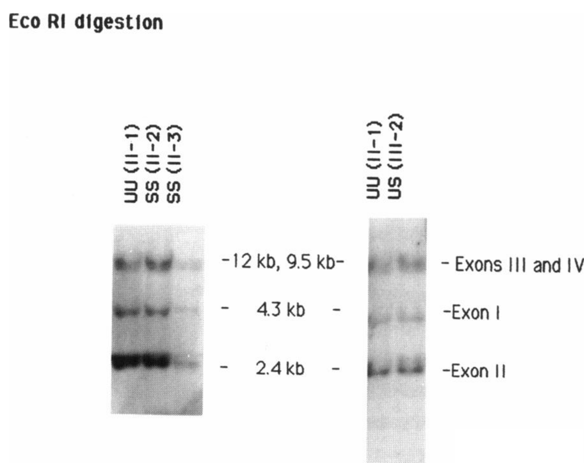

Figure 6 Genomic blots of DNA from UU, US, and SS individuals from family 1. *Eco*RI digestion yielded bands of 4.3, 2.4, 12, and 9.5 kb, with the hybridization probes containing, respectively, exons I, II, III, and IV of the serum cholinesterase gene.

Usual genotype GGT		Intermediate		New genotype GGAG
1) 5' GGT 3'	--- Transversion--->	GGA (Gly)	---------+G--------->	GGAG
2) 5' GGT 3'	---Transversion--->	GGG (Gly)	------- +A---------->	GGAG
3) 5' GGT 3'	------- +G---------->	GGTG (Gly+1bp)	---Transversion--->	GGAG
4) 5' GGT 3'	------- +A---------->	GGAT (Gly+1bp)	---Transversion--->	GGAG

Figure 7 Possibilities that could explain the complex mutation (transversion associated to a frameshift mutation) causing the silent genotype for human serum cholinesterase on variant Ann Arbor (Gly 117, GGT→GGAG).

Discussion

The Molecular Basis of the Mutation

Two events are necessary to change the sequence from GGT to GGAG, the codon for Gly 117 and site of the frameshift mutation in the silent phenotype: (1) the addition of one base and (2) a point mutation in another one. The transversion (GGT to GGA or GGG) is not relevant for the phenotype by itself, since all of the three codons would produce Gly at this position.

The defect primarily responsible for the silent phenotype is the change in the reading frame (+1) from Gly 117 until the stop codon is reached at codon 129. The appearance of this codon early in the sequence of the protein, before the active-site serine is reached, predicts that a short, nonfunctional polypeptide could be produced.

It is possible to speculate about the molecular basis of this defect. It is well established that the primary sequence in the DNA is very important in determining the frequency of frameshift errors. The classical model, a slippage mechanism (Streisinger et al. 1966), proposes that frameshift mutations are likely to occur in regions with repeating bases, base doublets (Okada et al. 1972), or tandemly repeated sequences (Farabaugh et al. 1978). By this model, the mispairing of the strands after the production of a gap in the DNA (e.g., during replication, recombination, or repair) would account for the insertion or deletion of bases, causing the frameshift. A clear role for DNA polymerase has been demonstrated in controlling the frequency and specificity of frameshift mutations, as has been shown by Kunkel (1986) in vitro for eukaryotic DNA polymerases from different animals.

It is reasonable to try to explain our results by this model, because the sequence of nucleotides from Gly 115→Gly 117 shows a tandemly repeated sequence of triplets 5′ TGG-TGG-TGG 3′, very similar to the "frameshift hotspot," 5′ CTGG-CTGG-CTGG 3′, described for the *lacI* gene of *Escherichia coli* (Farabaugh et al. 1978), which generates spontaneous frameshift mutations at a very high rate.

The slippage mechanism model also predicts that the base to be added or deleted is identical to an adjacent base within the sequence. On that basis, in the alteration to Gly 117 – GGT→GGAG – the extra base would be G, and A would be the point mutation, since there is no A in the sequence around this site.

Although this model offers a very attractive explanation for the addition of extra bases at this position, it does not give an obvious explanation for the association of the two mutations (the frameshift and the transversion) by a concerted event; possibly they occurred not at the same time but as two sequential events. Data from the literature show that the combination of point mutations and extra bases at the same site, such as the one we observed, are rarely seen: Farabaugh et al. (1978) reported that, in at least two systems, frameshift mutations by deletions were found in conjunction with point mutations, but this association seems to be the exception rather than the rule, for spontaneous frameshift mutations occurring in the *lacI* gene of *E. coli*. Frameshift mutations in humans are not ordinarily associated with point mutations at the same site (see Pihlajaniemi et al. 1984; Nukiwa et al. 1987; Chan et al. 1988; Kazazian and Boehm 1988; Sifers et al. 1988; Bateman et al. 1989; Endo et al. 1989; Miura et al. 1989).

If more than one event is responsible for the mutation we are reporting, there are four possibilities, if a minimal number of mutational events (i.e., two) are assumed (fig. 7). Possibilities 1 and 2 assume that in a

first step, Gly 117, GGT underwent a mutation to GGG or GGA, producing an intermediate still coding for glycine at this position. In the second step, an extra base was introduced. Possibilities 3 and 4 assume that the frameshift preceded the point mutation and that the transversion occurred afterward. All possibilities predict only transversions, not transitions.

Even though possibility 1 seems to be the most likely, we have no further evidence supporting this alternative; for example, we did not find GGA or GGG as alternative codons for Gly at this position and we also have no silent genotypes with the frameshift but without the transversion.

One mutation in this region was detected in our laboratory, in a DNA sample which did not represent the silent phenotype. It was a sample, amplified by PCR, which showed a point mutation at Gly 117, resulting in the abnormal sequence GGT-GGT-AGT (Gly-Gly-Ser for amino acids 115–117). This result was not confirmed by the direct sequencing of the DNA when another amplification mixture was used, and it appeared to result from an error made by the *Taq* DNA polymerase during amplification (F. Jensen, personal communication). Even though we have occasionally detected mistakes made by this enzyme in other positions, it raises the possibility that regions with repeating bases or with sequences such as this one may be preferential sites for mutations not only in vivo but also in vitro, during amplification with a DNA polymerase. The results emphasize the need to confirm any mutation by more than one amplification mixture when searching for genetic variants.

In conclusion, it is possible that mutations around Gly 117 in the gene of butyrylcholinesterase are particularly likely to occur, because of the tandem repeat. If so, an appreciable fraction of the silent phenotypes for serum cholinesterase may be explained by this mutation, although the possibility of a founder effect must also be considered. The number of individuals with silent phenotype from different families we have analyzed is still very small, and additional studies will be necessary to test this hypothesis.

The Silent Genotype

In the present report, all values for serum cholinesterase activity in both pedigrees (fig. 2) are fully compatible with the hypothesis of a silent allele segregating with the usual allele in family 1 and with the usual and atypical alleles in family 2.

The information obtained on a nucleotide level is particularly interesting in families such as family 2, in which no silent homozygotes are present, only heterozygotes for this condition. This family is like the two earlier reported families (Kalow and Staron 1957; Harris et al. 1960) in which the presence of a silent allele was proposed to explain the apparent discrepancy of phenotypes, in which the heterozygous mother (UA) and the usual homozygous father (UU) had two children that appeared to be atypical homozygotes (AA). The discrepancy disappears when the father is actually identified as being a heterozygous US and the two children are identified as heterozygotes, AS. The analysis of the atypical site at Asp 70 in the two children showed that they are, indeed, heterozygotes Asp/Gly at this position—not homozygotes Gly/Gly, as would be expected for AA individuals.

These observations demonstrate how the sequencing of the DNA confirmed the genotype predicted from the characteristics of the serum enzyme. It is also possible to predict that, in addition to the homozygotes for the silent allele of family 1, both the AS heterozygotes in family 2 (II-1 and II-2) are predisposed to prolonged muscle relaxation (including apnea) if given succinylcholine, not only because they have low activity of serum cholinesterase but also because all the activity present is atypical, with a lower affinity for succinylcholine.

Many cases of silent phenotypes for serum cholinesterase have been reported, and analysis of these individuals at the nucleotide level is essential for identifying the different types of defects, as well as for classifying individuals within this heterogeneous phenotype. The serum with silent phenotype used as a control in the rocket immunoelectrophoresis (fig. 5) presents the first evidence of molecular heterogeneity within the silent phenotype. Even though only a small portion of the DNA of this individual has been sequenced, the possibility of a frameshift mutation in the same position has been excluded. Thus, another mechanism must account for the absence of enzymatic activity in that serum.

The variant described in the present report would be classified as belonging to the type I category proposed by Rubinstein et al. (1970), i.e., silent phenotype with no cross-reactive material. The designation of the variant described in the present paper in accordance with the new nomenclature proposed for the genetic variants of serum cholinesterase (B. La Du, unpublished data) would be "BCHE*FS117," because the variation in the locus of butyrylcholinesterase (serum cholinesterase) is caused by a frameshift at amino acid 117. We suggest that this mutation be designated "variant Ann Arbor," as a trivial name based on the name of the city in which the family 1 members reside.

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