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# Identification of Two Different Point Mutations Associated with the Fluoride-resistant Phenotype for Human Butyrylcholinesterase

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## Summary

**The fluoride variant of human butyrylcholinesterase owes its name to the observation that it is resistant to inhibition by 0.050 mM sodium fluoride in the in vitro assay. Individuals who are heterozygous for the fluoride and atypical alleles experience about 30 min of apnea, rather than the usual 3–5 min, after receiving succinyldicholine. Earlier we reported that the atypical variant has a nucleotide substitution which changes Asp 70 to Gly. In the present work we have identified two different point mutations associated with the fluoride-resistant phenotype. Fluoride-1 has a nucleotide substitution which changes Thr 243 to Met (ACG to ATG). Fluoride-2 has a substitution which changes Gly 390 to Val (GGT to GTT). These results were obtained by DNA sequence analysis of the butyrylcholinesterase gene after amplification by PCR. The subjects for these analyses were 4 patients and 21 family members.**

## Introduction

The drug succinyldicholine is given to surgery patients to facilitate tracheal intubation. In 99% of all patients the effect of this muscle relaxant disappears in 3–5 min. However, some patients are unable to breathe for as long as 2 h after a single dose of succinyldicholine. Kalow and Gunn (1957) were the first to recognize that the abnormal response was due to an inherited alteration in serum butyrylcholinesterase (E.C.3.1.1.8; serum cholinesterase, pseudocholinesterase). Further developments in phenotyping assays have resulted in the recognition of at least six different genetic variant forms of butyrylcholinesterase, including atypical, silent, fluoride, H, J, and K variants (reviewed by Whittaker 1986). The point mutations present in the coding regions of the BCHE gene of many of these variants have recently been identified by our laboratory (McGuire et al. 1989; Nogueira et al.1990; Bartels et al. 1992*a*, 1992*b*). A silent variant in which the BCHE gene contains a 342-bp insertion has been identified by Muratani et al. (1991). In addition we have found one point mutation in the 5′ untranslated region and one in the 3′ untranslated region (Bartels et al. 1990, 1992*b*). In the present report we show that two different point mutations give the fluoride-resistant phenotype of butyrylcholinesterase.

The fluoride variant of human butyrylcholinesterase was discovered by Harris and Whittaker in 1961. Since then, many laboratories have confirmed the existence of a fluoride variant (Lehmann et al. 1963; Dietz et al. 1972; Viby-Mogensen and Hanel 1978; Alcantara et al. 1991), and the clinical observation is firmly established that heterozygotes with the AF (atypical/fluoride) phenotype have a moderately prolonged response to the muscle relaxant succinyldicholine (Viby-Mogensen 1981). These observations contrast with the clinical finding for heterozygotes with the UA (usual/atypical) phenotype, who most often have a

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normal response to succinyldicholine (Lehmann et al. 1963; Viby-Mogensen and Hanel 1978). People with the AF phenotype have apnea of shorter duration than do people with the AA (atypical/atypical) phenotype. We were interested in finding the structural alterations in the fluoride phenotype as a first step toward understanding alterations in enzyme activity.

## Materials and Methods

### Blood Samples

During the past 30 years two of our laboratories (B.N.L. and H.M.R.) routinely determined butyrylcholinesterase phenotypes on serum and plasma samples sent to us by anesthesiologists. About 2,500 samples were tested. For the present study we chose several families from this collection who carried the fluoride variant. We obtained 10–20 ml of fresh whole blood from 25 members of these families.

### Phenotyping Assays

Plasma butyrylcholinesterase activity was measured with benzoylcholine (Kalow and Lindsay 1955). Phenotype was determined by measuring dibucaine number (DN) (Kalow and Genest 1957), fluoride number (FN) (Harris and Whittaker 1961), and Roche number (RoN) (Evans and Wardell 1984).

### PCR

Genomic DNA was prepared from white blood cells (Maniatis et al. 1982), and 1–2 μg were amplified by PCR (Saiki et al. 1988). Amplification primers corresponded to the human BCHE gene (Arpagaus et al. 1990). Amplification primers have been described by McGuire et al. (1989) and Bartels et al. (1992*b*). PCR samples were sequenced directly using internal 20-mer primers labeled at the 5′ end (McGuire et al. 1989). Figure 1 indicates the location of the amplification and sequencing primers in the BCHE gene.

### Restriction-Enzyme Analysis

A *Mae*II restriction site, ACGT, is lost in the fluoride-1 variant. An *Hph*I site, GGTGA(N)$_8$, is lost in the fluoride-2 variant. DNA to be tested for the fluoride-1 point mutation was amplified with primers AP1/AP23 or AP3/AP23 and was digested with *Mae*II (Boehringer Mannheim). DNA to be tested for the fluoride-2 point mutation was amplified with primers AP21/AP4 and was digested with *Hph*I (New England Biolabs). The BCHE gene from all individuals

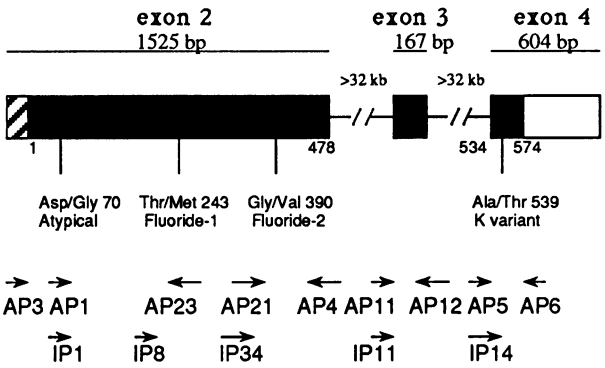


**Figure 1** Partial schematic representation of the gene for human butyrylcholinesterase, showing the location of amplification and sequencing primers. Exons are represented as bars: the coding sequence is solid black, the 28-amino-acid signal peptide is a striped box, and the 3′ untranslated region following the C-terminal amino acid 574 is a white box. Amino acid 1 is Glu at the N-terminus of mature, secreted butyrylcholinesterase; 478 is the last amino acid of exon 2; 534 is the first amino acid of exon 4; and 574 is the C-terminus. Two introns of about 32 kb are indicated. Several of the amplification primers (AP) and internal primers (IP) for sequencing are indicated. The atypical point mutation changes Asp 70 to Gly; fluoride-1 changes Thr 243 to Met; fluoride-2 changes Gly 390 to Val; and K-variant changes Ala 539 to Thr.

in pedigrees A and C, as well as sample D, was analyzed for the *Hph*I restriction site. Digests were analyzed on 3% agarose gels stained with ethidium bromide. Amplification primers for these experiments were AP1, 5′CTTGGTAGACTTCGATTCAAAAAGCCACAGTCTCT; AP3, 5′GCAAAGTCACAATCATATGCATCAGATTTCTCTTT; AP4, 5′AAGCCAGAGAACAATGACAAAAAATCAGCACTTAC; AP21, 5′GCTTTTTTAGTCTATGGTGCTCCTGGCTTCAGCAAAG; and AP23, 5′ GGGACAACAAATGCTTCATTCAGAAGAATTTCTTGGGGA.

### Nomenclature

The human genome contains a single copy of the BCHE gene (Arpagaus et al. 1990; Allderdice et al. 1991; Gaughan et al. 1991). BCHE is located on chromosome 3q26. Therefore the old nomenclature—"$E_1$" and "$E_2$," referring to two genes—is no longer appropriate.

La Du et al. (1991) have proposed a new system of trivial names to reflect the fact that multiple point mutations exist on one allele. For example, in the name "AK/F," a slash separates two alleles; one allele has the two mutations A (atypical) and K (K variant), while the second allele has only the F (fluoride) mutation. We continue to use the traditional names and

symbols for the atypical, K variant, and fluoride variant because they have been used to report phenotyping results for over 100,000 persons (Whittaker 1986). The formal name for the fluoride-1 allele is BCHE*243M, and that for the fluoride-2 allele is BCHE*390V. Formal names for genotypes are too long for routine use; for example, the formal name for the AK/F-2 genotype is BCHE*70G539T/BCHE*390V (La Du et al. 1991).

## Results

### *The Fluoride-1 Mutation*

The first mutation associated with the fluoride phenotype is a transition of C to T, which changes Thr 243 (ACG) to Met 243 (ATG) (fig. 2). This mutation was found in two members of the family shown in figure 3. Phenotype assays had predicted that both people were carriers of the fluoride-resistant gene. DNA sequencing of the BCHE gene in these two people revealed two additional mutations, but these additional mutations had been recognized earlier as the atypical variant linked to the K variant (McGuire et al. 1989; Bartels et al. 1992*b*). Carriers of the fluoride-1 mutation had the normal sequence (McTiernan et al. 1987) in the region of the fluoride-2 site, at Gly 390. Therefore there is no linkage between the fluoride-1 and fluoride-2 mutations. The AK allele segregated independently of the fluoride-1 allele, suggesting that the AK mutations were not linked to the fluoride-1 mutation.

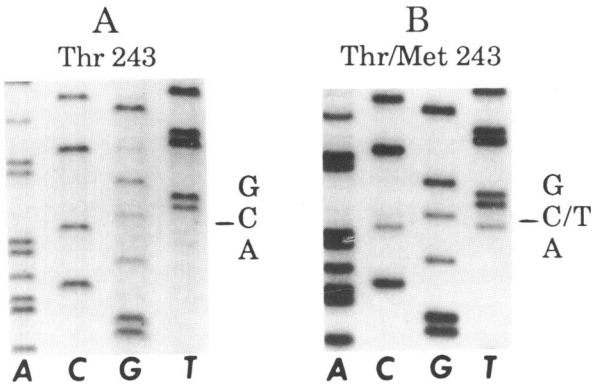


**Figure 2** Fluoride-1 mutation identified on DNA sequencing gels. *A*, Sequence of the fluoride-1 site in an individual with the usual type of butyrylcholinesterase (i.e., Thr 243 ACG). *B*, Sequence of the fluoride-1 site in a heterozygous carrier of the fluoride-1 mutation, showing both Thr 243 ACG and Met 243 ATG.

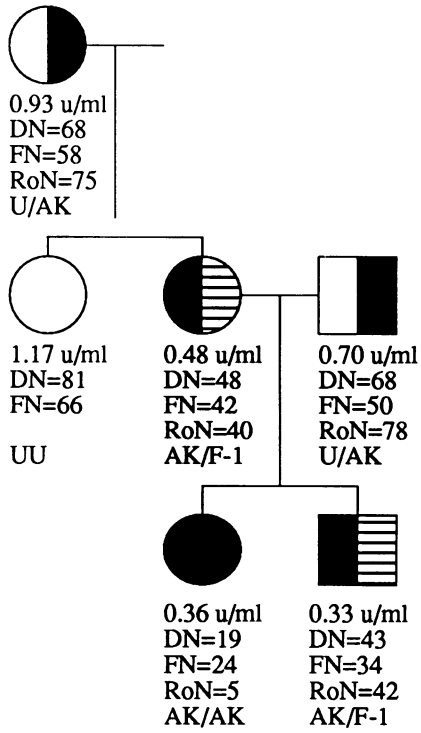


**Figure 3** Segregation of the fluoride-1 allele of butyrylcholinesterase. Genotypes are indicated as follows: stripes denote fluoride-1 (F-1); black denotes atypical linked to K variant (AK); and white denotes usual (U). Genotype was determined by DNA sequencing. The trivial name for the genotype is given below the phenotype data on each individual. Phenotype results are given as units of activity per milliliter (u/ml), dibucaine number (DN), fluoride number (FN), and Roche number (RoN).

The results observed by DNA sequencing were confirmed by restriction-enzyme analysis. Figure 4 shows that PCR fragments digested with *Mae*II gave bands that were consistent with both the presence of the fluoride-1 mutation in genotype AK/F-1 and the absence of the fluoride-1 mutation in genotypes AK/F-2 and U/AK.

### *The Fluoride-2 Mutation*

The second mutation associated with the fluoride phenotype is a transversion of G to T, which changes Gly 390 (GGT) to Val 390 (GTT) (fig. 5). This mutation was found in 10 people from four different families (fig. 6). The hereditary nature of the fluoride-2 mutation is most apparent in families A and B in figure 6, where three generations carry the same mutation.

Carriers of the fluoride-2 mutation had the normal sequence at the fluoride-1 site, at Thr 243. Thus, the

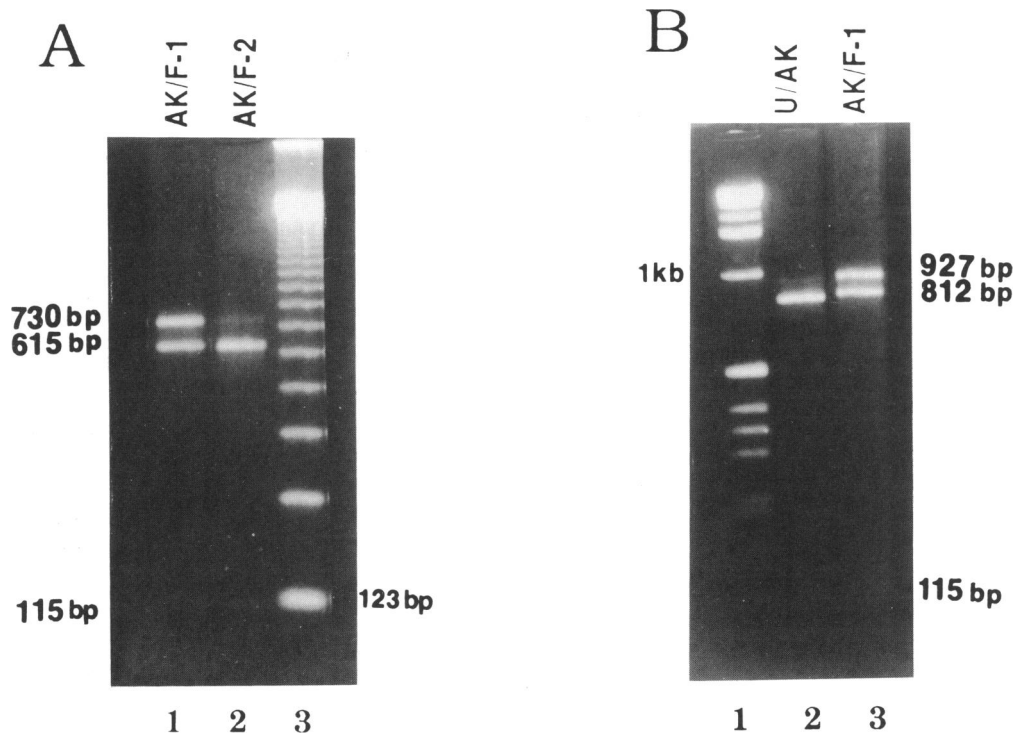


**Figure 4** Detection of the fluoride-1 heterozygote by digestion of amplified DNA with *Mae*II. *A,* Genomic DNA amplified with primers AP1/AP23 to give a 730-bp fragment. The 730-bp fragment was digested with *Mae*II and separated on 3% agarose. Lane 1, DNA from genotype AK/F-1 (i.e., the propositus) which has the 730-bp band characteristic of the F-1 variant because of loss of the *Mae*II site; it also has 615-bp and 115-bp bands, as expected, from the normal allele. Lane 2, DNA from genotype AK/F-2, which has the normal pattern (615 bp and 115 bp only). Lane 3, Molecular-weight marker, 123-bp ladder. *B,* Genomic DNA from the father and mother of the propositus, amplified with primers AP3/AP23 to give a 927-bp fragment. The 927-bp fragment was digested with *Mae*II and separated by agarose gel electrophoresis. Lane 1, Molecular-weight marker, 1-kb ladder. Lane 2, DNA from genotype U/AK, father of the propositus, which has the normal pattern (812 bp and 115 bp only). Lane 3, DNA from genotype AK/F-1, mother of the propositus, which has the 927-bp band characteristic of the F-1 variant because of loss of the *Mae*II site; it also has the expected 812-bp and 115-bp bands arising from the normal allele.

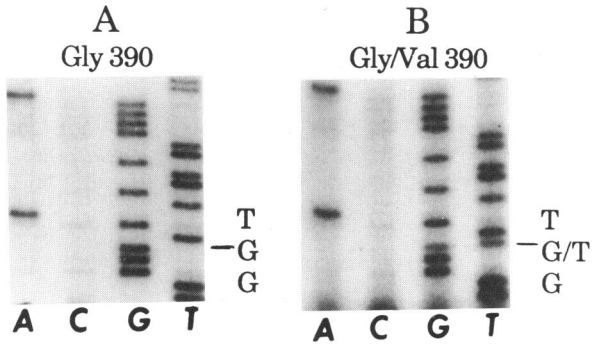


**Figure 5** Fluoride-2 mutation identified on DNA sequencing gels. *A,* Sequence of the fluoride-2 site in an individual with the usual type of butyrylcholinesterase (i.e., Gly 390 GGT). *B,* Sequence of the fluoride-2 site in a heterozygous carrier of the fluoride-2 mutation, showing both Gly 390 GGT and Val 390 GTT.

fluoride-2 mutation is not linked to the fluoride-1 mutation. Nor is the fluoride-2 mutation linked to the atypical, the K variant, or the J variant mutations (Bartels et al. 1992*a*), despite the high incidence of the A and K variant mutations shown in figure 6. The high number of additional mutations is a consequence of the fact that the samples were chosen on the basis of the phenotyping assay of Harris and Whittaker (1961), which can detect the fluoride variant only in heterozygous combination with the atypical variant. Family A in figure 6 does not show a person with the AF phenotype, because we were unable to obtain a fresh sample of blood from this person. However, family A was originally identified as a family that carried the fluoride-resistant allele through a family member with the AF phenotype.

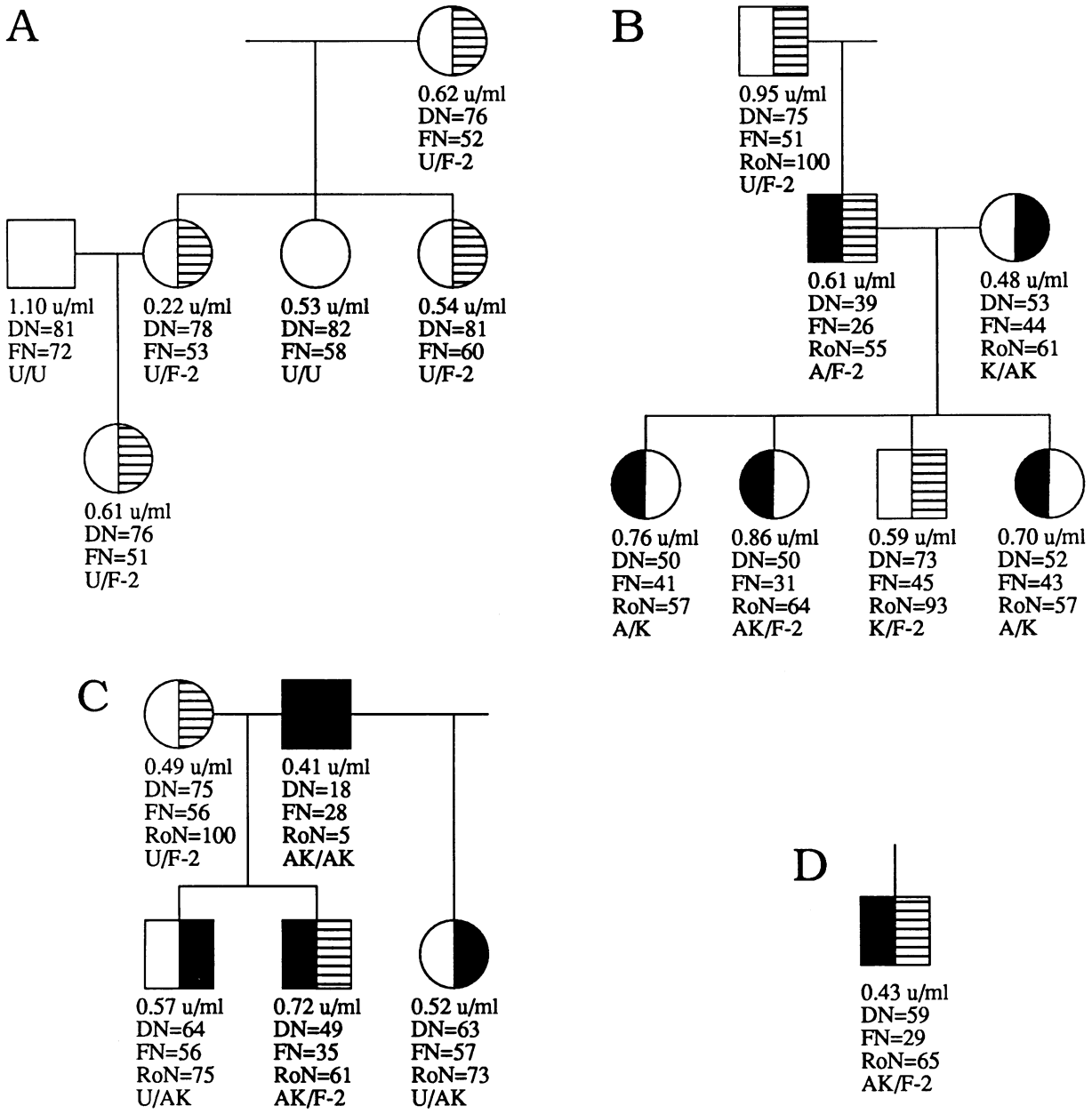


**Figure 6** Three pedigrees and one individual, all segregating the fluoride-2 variant. Genotypes are indicated as follows: stripes denote fluoride-2 (F-2); black denotes atypical (A) or atypical linked to K variant (AK); and white denotes usual (U) or K variant (K). Genotype was determined by DNA sequencing. Samples from family A were not sequenced in the region of the K variant. The trivial name for the genotype is given below the phenotype data on each individual. Phenotype results are given as units of activity per milliliter (u/ml), dibucaine number (DN), fluoride number (FN), and Roche number (RoN).

As shown in figure 7 the presence of the fluoride-2 mutation was confirmed by restriction-enzyme digestion of PCR-amplified material. The fragments showed the disruption of the *Hph*I site in carriers of the fluoride-2 allele predicted from the sequence.

Genomic DNA from genotype U/F-2 was digested with *Eco*RI and was analyzed on a Southern blot (data not shown; but similar results have been reported by Nogueira et al. [1990]). The results showed the normal pattern of bands (12, 9, 4.3, and 2.4 kb) with

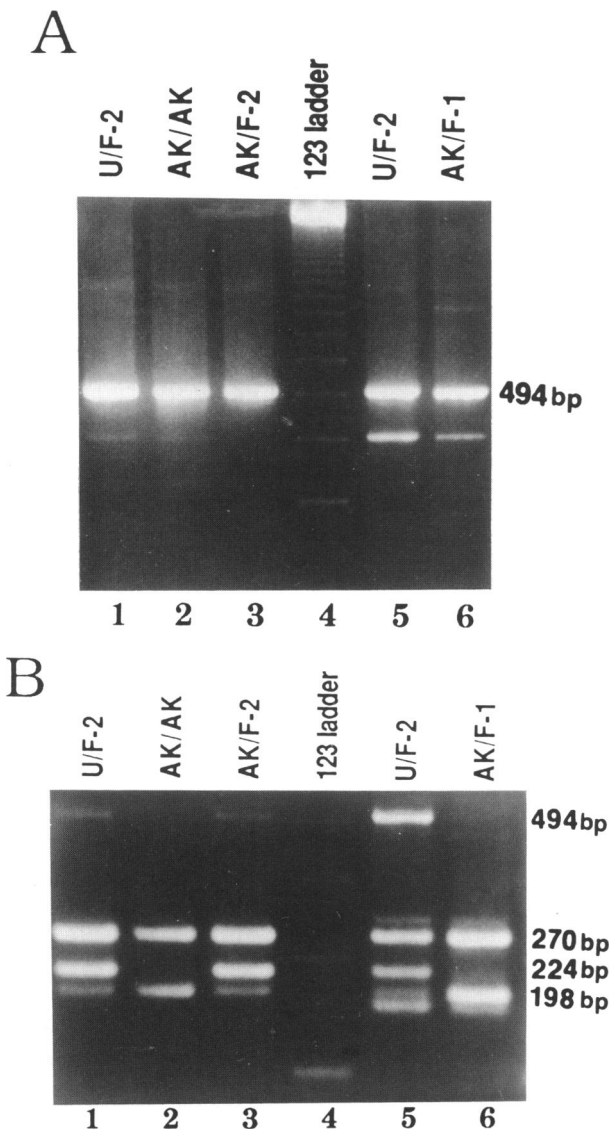


**Figure 7** Detection of the fluoride-2 heterozygote by digestion of amplified DNA with *Hph*I. *A*, Genomic DNA from five people was amplified with primers AP21/AP4 to give a 494-bp fragment. The agarose gel shows that the undigested product gave a major 494-bp band and a minor contaminating band. The molecular-weight marker in lane 4 is the 123-bp ladder. *B*, 494-bp piece digested with *Hph*I and separated by agarose gel electrophoresis. Two *Hph*I sites exist. One site yields a 270-bp and a 224-bp piece. The 270-bp band is common to all samples. The 224-bp piece contains the second *Hph*I site which is eliminated by the F-2 mutation. Digestion at this site yields a 198-bp and a 26-bp piece. Lanes 1, 3, and 5 show that genotypes U/F-2 and AK/F-2 have both the 224-bp band characteristic of the F-2 variant and the 198-bp band arising from the other normal allele. Lanes 2 and 6 show that genotypes AK/AK and AK/F-1 have only those bands which come from the normal sequence (270 bp and 198 bp). The 26-bp band is not visible in this system.

normal intensities. This suggests that the BCHE gene is neither rearranged nor amplified in the fluoride-2 variant.

## Discussion

We have found two point mutations in the BCHE gene of people who have the fluoride phenotype. Fluoride-1 (ACG to ATG) changes Thr 243 to Met. Fluoride-2 (GGT to GTT) changes Gly 390 to Val. The crystal structure of *Torpedo* acetylcholinesterase (Sussman et al. 1991) shows that Thr 243 and Gly 390 are in two different alpha helixes. Comparison of amino acid sequences (fig. 8) shows conservation at Thr 243 in the butyrylcholinesterases but not in the acetylcholinesterases. Gly 390 is not conserved between species. Kinetic studies of the fluoride-2 variant (Masson et al., in press) indicate that Gly 390 is important for binding of substrates and ligands. Thus, the clinical effect of succinyldicholine in patients carrying the fluoride-2 variant may be explained in terms of reduced binding affinity for succinyldicholine.

The fluoride-1 protein has a second structural alteration in addition to the amino acid substitution. The mutation changing Thr 243 to Met decreases the number of carbohydrate chains per subunit, from nine to eight. A carbohydrate chain is normally attached to Asn 241 (Lockridge et al. 1987). This was established during amino acid sequencing by observing the exact location of each sugar chain. Thr 243 is part of the Asn-X-Thr/Ser tripeptide recognition sequence for

| | | CHO | 243 | | | | | | | 390 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Human | BCHE | N | R | **T** | L | N | . | . | . | A | L | **G** | D | V |
| Mouse | BCHE | N | R | T | L | T | . | . | . | A | L | D | D | V |
| Rabbit | BCHE | N | R | T | L | T | . | . | . | A | L | A | A | V |
| Human | ACHE | R | R | A | T | Q | . | . | . | A | L | S | D | V |
| Bovine | ACHE | R | R | A | T | L | . | . | . | A | L | S | D | V |
| Mouse | ACHE | R | R | A | T | L | . | . | . | A | M | S | A | V |
| Torpedo | ACHE | R | R | A | V | E | . | . | . | G | L | D | D | I |
| Drosoph | ACHE | E | I | G | K | A | . | . | . | Q | I | G | R | A |
| Mosquito | ACHE | Q | I | A | E | G | . | . | . | Q | V | G | R | A |

**Figure 8** Amino acid sequence comparisons in the regions of the fluoride-1 and fluoride-2 mutations in human BCHE. The fluoride-1 mutation changes Thr 243 to Met. The fluoride-2 mutation changes Gly 390 to Val. CHO = carbohydrate chain attached to Asn 241 in human, mouse, and rabbit BCHE. Sequences are from Lockridge et al. (1987), for human BCHE; Rachinsky et al. (1990), for mouse BCHE and ACHE; Jbilo and Chatonnet (1990), for rabbit BCHE; Soreq et al. (1990), for human ACHE; Doctor et al. (1990), for bovine ACHE; Schumacher et al. (1986), for *Torpedo* ACHE; Hall and Spierer (1986), for *Drosophila* ACHE; and Hall and Malcolm (1991), for mosquito ACHE.

glycosylation. Without Thr 243, Asn 241 cannot be glycosylated. The nine carbohydrate chains account for 23.9% of the weight of butyrylcholinesterase. Loss of one carbohydrate chain is expected to reduce the molecular weight by 2.7%, so that the subunit weight is expected to be 82,700 rather than 85,000.

The fluoride-1 mutation seems to follow a relatively common mechanism underlying genetic variants in human subjects. The C in a CpG dinucleotide is methylated and then deaminated to form a T. The T is not efficiently corrected, resulting in a C-to-T transition (Cooper and Youssoufian 1988).

Our 30-year collection of butyrylcholinesterase variants contained not a single homozygous fluoride variant, insofar as we could judge from phenotyping results. That is why our studies were done on heterozygotes. The homozygous fluoride variant occurs in 1 of 150,000 persons (Whittaker 1986), a frequency similar to that of the homozygous silent variant, and yet our collection contained six homozygous silent samples. The likely explanation is that some of the samples phenotyped as AF are actually FF; it is easy to confuse the two in phenotyping assays, since they have similar DN and FN. The techniques described in the present report will remove the ambiguity from analysis of future samples.

We have expressed homozygous fluoride-2 butyrylcholinesterase by recombinant DNA technology (Adkins et al. 1991; Masson et al., in press). The secreted enzyme had a DN of 66, FN of 36, and RoN of 88. These values are in agreement with phenotype assay results (DN = 64–68; FN = 34–35; and RoN = 75–86) for the three published homozygous fluoride samples (Lehmann et al. 1963; Liddell et al. 1963; Whittaker 1964) and support our assignment of the mutation at glycine 390 to the fluoride-resistant phenotype.

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