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Genetics

An unusual insertion/deletion in the gene encoding the β-subunit of propionyl-CoA carboxylase is a frequent mutation in Caucasian propionic acidemia

(polymerase chain reaction/allele-specific oligonucleotide hybridization/genetic heterogeneity/interallelic complementation/*PCCB* gene)

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ABSTRACT **Propionic acidemia is an inherited disorder of organic acid metabolism that is caused by deficiency of propionyl-CoA carboxylase (PCC; EC 6.4.1.3). Affected patients fall into two complementation groups, *pccA* and *pccBC* (subgroups *B*, *C*, and *BC*), resulting from deficiency of the nonidentical α and β subunits of PCC, respectively. We have detected an unusual insertion/deletion in the DNA of patients from the *pccBC* and *pccC* subgroups that replaces 14 nucleotides in the coding sequence of the β subunit with 12 nucleotides unrelated to this region of the gene. This results in elimination of an *Msp* I restriction site, a 2-base-pair (bp) deletion, a frameshift, and a stop codon in the new frame ≈100 amino acid residues proximal to the normal carboxyl terminus. Among 14 unrelated Caucasian patients in the *pccBC* complementation group, this unique mutation was found in 8 of 28 mutant alleles examined. Mutant allele-specific oligonucleotide hybridization to amplified genomic DNAs revealed that the inserted 12 nucleotides do not originate in an ≈1000-bp region around the mutation. In the course of our investigation, we identified another mutation in the same exon: a 3-bp in-frame deletion that eliminates one of two isoleucine codons immediately preceding the *Msp* I site. Two unrelated patients were compound heterozygotes for this single-codon deletion and for the insertion/deletion described above. We conclude that either there is a propensity for the PCC β-subunit gene to undergo mutations of this sort at this position or, more likely, the mutations in all of the involved Caucasian patients have a common origin in preceding generations.**

Propionyl-CoA carboxylase [PCC; propanoyl-CoA:carbon-dioxide ligase (ADP-forming), EC 6.4.1.3] is a key enzyme in the catabolic pathway for odd-chain fatty acids, isoleucine, threonine, methionine, and valine (1). Native PCC is an oligomer composed of nonidentical subunits (α and β); the required cofactor, biotin, is bound covalently to the α subunit. The apparent native molecular weight of the human enzyme is ≈540,000; the α and β subunits have molecular weights of 70,000–72,000 and 54,000–56,000, respectively (2). Other evidence suggests that the enzyme has an $(\alpha\beta)_6$ subunit composition (3). In humans, deficiency of PCC activity is inherited as an autosomal recessive trait; the disease shows considerable clinical heterogeneity but often causes life-threatening ketoacidosis in neonates. The clinical manifestations of PCC deficiency can be controlled in most affected children by strict limitation of dietary protein intake to avoid recurrent acidotic episodes.

Patients can be classified into two major intergenic complementation groups, *pccA* and *pccBC*, corresponding to the genes encoding the α and β subunits, respectively. The *pccBC* group is further subdivided into *pccBC*, *pccB*, and *pccC* subgroups, the latter two subgroups reflecting interallelic complementation (4). Full-length cDNAs for the α and β subunits have been cloned in our laboratory from rat liver (5, 6). The isolation of human cDNA clones encoding the α subunit (7, 8) and a portion of the β subunit has been reported (7). The corresponding genes, *PCCA* and *PCCB*, have been mapped to chromosomes 13 and 3, respectively (7, 9). Northern blot analysis revealed the absence of α mRNA (2.8–2.9 kilobases) in most *pccA* patients (10, 11) and greatly reduced amounts of β mRNA (2.0 kilobases) in *pccBC* patients (11). Western blot analysis showed the absence of both α and β subunits in *pccA* patients (11); in *pccBC* patients, however, the β subunit was usually and selectively reduced in amount and often in size.

We now present information concerning the molecular basis for PCC β-subunit (βPCC) deficiency in some patients. We have found two different mutations in the same exon of *PCCB*: (*i*) a frequent and unusual insertion/deletion and (*ii*) an infrequent single-codon deletion.‡

MATERIALS AND METHODS

Cell Lines and Cell Culture. The cell lines used were cultured skin fibroblasts from 20 controls, 7 *pccA* patients, 3 *pccB* patients [68, 1750, and 1751 (Yale cell-line accession numbers)], 3 *pccBC* patients (519, 534, and 1747), and 9 *pccC* patients (148, 269, 467, 543, 572, 581, 633, 756, and 1752). Conditions for culturing and harvesting cells were as described (12).

Isolation of DNA and Southern Blot Analysis. High molecular weight DNA was isolated from cultured skin fibroblasts according to a published method (13). Eight micrograms of genomic DNA from each cell line was digested with the restriction endonuclease *Msp* I (New England Biolabs), electrophoresed, blotted, and hybridized at 65°C as reported (14). The *Pst* I fragment [0.8 kilobase pair (kbp)] from the human βPCC cDNA (7) was used as the *PCCB* gene probe.

Isolation of Total RNA and Reverse Transcription. One or two roller bottles (850 cm^2; Falcon) containing confluent monolayers of each cell line were harvested by trypsinization and stored frozen at −70°C. Total RNA was prepared as described (15) from 400–500 μg of cell pellet. Reverse transcription was carried out in 50-μl reaction mixtures as described (9) with 5 μg of total RNA and an oligo(dT) primer (40 μg/ml). An aliquot (15 μl) of the reaction mixture was used directly for amplification.

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Abbreviations: PCC, propionyl-CoA carboxylase; βPCC, PCC β subunit; PCR, polymerase chain reaction; ASO, allele-specific oligonucleotide.

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‡The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M31167–M31169).

Polymerase Chain Reaction (PCR) Amplification. PCR was carried out in 100-μl reaction mixtures employing the standard conditions (16): 10 mM Tris Cl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, gelatin at 100 μg/ml, and 0.2 mM deoxynucleoside triphosphates. Two primers, 600–800 ng each, were added, and 800–1000 ng of genomic DNA or the cDNA in 15 μl of reverse transcription mixture was used as template. For genomic DNAs, 2.5 units of *Thermus aquaticus* (*Taq*) DNA polymerase (Perkin–Elmer/Cetus) was added after the mixtures had been incubated at 65°C for 5 min. For the amplification of cDNA, the cDNA was added to the PCR reaction mixtures after it had been heated at 95°C for 2 min, and then 2.5 units of *Taq* polymerase was added. The reaction mixtures were overlaid with 100 μl of mineral oil, and the temperature cycling (40 cycles) was carried out: for genomic DNAs, in a DNA Thermal Cycler (Perkin–Elmer/Cetus), 1 min at 94°C, 2 min at 55°C, and 1 min at 72°C; for cDNAs, in a Programmable Dri-Block PHC-1 (Techne, Princeton, NJ), 2 min at 94°C, 1 min at 37°C, and 2 min at 72°C.

PCR Primers. Fig. 1 shows the location of the primers on the partial βPCC cDNA sequence. Their DNA sequences were as follows: primer 1 (sense), 5′-TTCAATATTCCACTCATCACTTTTG-3′; primer 2 (antisense), 5′-AGGCCTTCCTGGTGATGACTGTGAC-3′; primer 3 (sense), 5′-GCCAAGCTTCTCTACGCATTT-3′; primer 4 (antisense), 5′-ACAAAGGTGCTTAGAGCTCAT-3′; primer 5 (sense), 5′-GGAATTGTTGGCAACCAACCTAAG-3′.

To facilitate subcloning of the PCR products, primer 1 and primer 5 contained (at the 5′ end) an additional 15 nucleotides (CGTAGGATCCGCGAA) coding for artificial *Bam*HI and partial *Eco*RI sites and an additional 10 nucleotides (CGTAGAATTC) coding for an artificial *Eco*RI site, respectively, while primer 2 contained (at the 5′ end) 16 additional nucleotides (CGCTCGAGCGATCGAT) encoding *Xho* I and *Cla* I sites. Primers 3 and 4 contained internal *Hin*dIII and *Sac* I sites, respectively.

Mutant Screening by Allele-Specific Oligonucleotide (ASO) Hybridization. PCR-amplified DNAs were denatured by boiling for 3 min and were electrophoresed in a 6% polyacrylamide/8 M urea gel. The DNA was electrophoretically transferred onto a Hybond-N membrane (Amersham). The membrane was dried at room temperature and baked under vacuum at 80°C for 2 hr. After prehybridization at 65°C, the oligonucleotide hybridization was performed at 37°C overnight, using the same solution as for the Southern blot analysis. The membrane was washed in 0.9 M NaCl/0.06 M sodium phosphate, pH 7.4/6 mM EDTA/1 mM sodium pyrophosphate at room temperature for 30 min. The washing temperature was gradually increased to 53°C to distinguish between perfect matches and mismatches.

DNA Sequencing. Direct sequencing of PCR-amplified DNAs was carried out by chemical cleavage (17). The DNA was radiolabeled in the PCR mixture by using one primer unlabeled and the other labeled at its 5′ end with [γ-^{32}P]ATP. Prior to sequencing, the amplified products were purified by ultracentrifugation (Centricon-30 microconcentrator; Amicon) and by electrophoresis in an acrylamide/glycerol gel [5% acrylamide/0.25% *N,N′*-methylenebisacrylamide/25% (vol/vol) glycerol], followed by electroelution (18).

In order to subclone the PCR-amplified products, the amplification reactions were performed with primers that contained artificial restriction endonuclease sites at their 5′ ends: *Eco*RI site, primer 1 or primer 5; *Cla* I site, primer 2. The PCR-amplified DNA was digested with *Eco*RI and *Cla* I and was ligated into an *Eco*RI/*Cla* I-cut pBluescript vector (Stratagene). Dideoxynucleotide chain-termination sequencing was carried out as described (19).

RESULTS

Southern Blot Analysis. Southern blot analyses of genomic DNAs digested with *Msp* I and probed with a portion of the human βPCC cDNA (probe A in Fig. 1; 800 bp) revealed a unique band (2.7 kbp) in DNAs from each of 3 *pccBC* patients (519, 534, and 1747) and from 5 of 9 *pccC* patients (467, 543, 572, 756, and 1752), two of whom (572 and 756) were siblings. DNAs from 20 control subjects, 7 *pccA* patients, and 3 *pccB* patients did not show this band (Fig. 2). Furthermore, the two affected siblings who showed this unique band appeared to be homozygous for this allele; i.e., they had neither the 2.1-kbp nor the 0.6-kbp band seen in controls. The observed mutant allele frequency in the affected *pccBC* group was 0.29 (8 out of 28 alleles).

To narrow down the region of the cDNA that detected the 2.7-kbp band, we hybridized the same blot with shorter probes derived from the cDNA. We determined that two adjacent portions of the cDNA, *Ban* II–*Sac* I (probe B in Fig. 1; 210 bp) and *Sac* I–*Pst* I (probe C in Fig. 1; 150 bp), detected the band. Because of the close proximity of the *Msp* I site to the *Sac* I site in the former fragment, we digested genomic DNA with both *Msp* I and *Sac* I to help localize the mutation. Under these

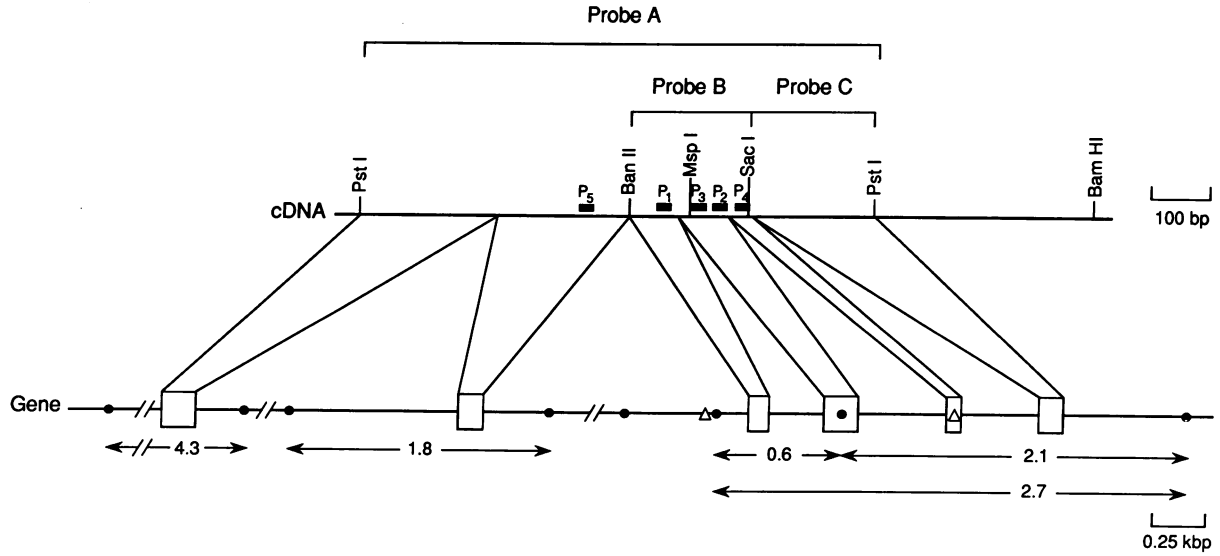

FIG. 1. Partial restriction maps of the βPCC cDNA and the corresponding region of the *PCCB* gene. The three cDNA probes (A, B, and C), the positions of the primers, and the location on the genomic map of the fragments seen on the Southern blot are indicated. P_1, P_2, P_3, P_4, and P_5 are PCR primers 1–5 (see *Materials and Methods*). *Msp* I and *Sac* I sites are shown on the genomic map (● and △, respectively).

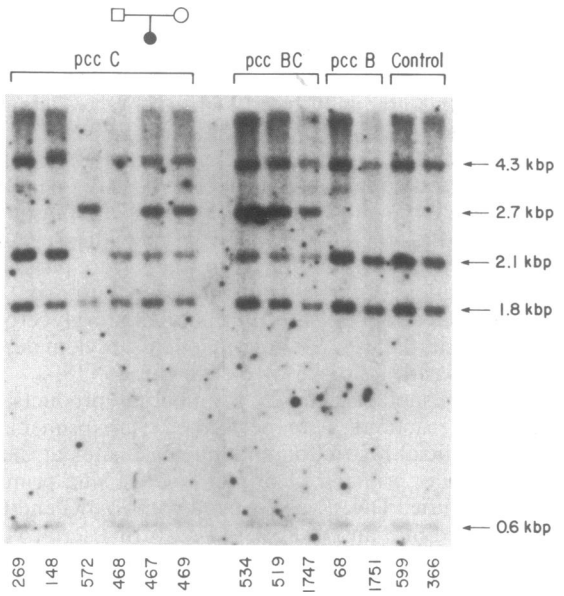

FIG. 2. Southern blot analysis of *Msp* I-digested genomic DNA from patients and controls hybridized with probe A (Fig. 1). The 2.7-kbp band is due to the lack of an *Msp* I site in some of the *pccC* and *pccBC* patients. One of the *pccC* patients (third lane from the left) is homozygous for this allele, because he lacks the 2.1- and 0.6-kbp bands.

conditions, we observed a new band (1.2 kbp) that hybridized only with the *Ban* II–*Sac* I fragment (data not shown). Thus, the particular *Msp* I site involved is the one located ≈300 bp 5′-wards of the stop codon in the βPCC cDNA.

PCR Amplification of Genomic DNAs. We amplified this region from genomic DNA by PCR with two primers (P_1 and P_2, Fig. 1) flanking the *Msp* I site. The size of the amplified product predicted from the cDNA was 150 bp; however, PCR amplification of genomic DNA yielded a 390-bp product, suggesting the presence of a small (240-bp) intron between the two primers, in agreement with the map derived from the Southern blots.

As expected, the PCR-amplified product from control DNA was nearly completely digested with *Msp* I, while about half of the product from patients heterozygous for the mutant allele was digested. Moreover, the product from the two *pccC* patients homozygous for this allele showed no digestion with *Msp* I (results not shown). These results provided further evidence that the *Msp* I site in this region of the coding sequence is the one altered in these patients.

Insertion/Deletion. We sequenced the PCR-amplified genomic DNA from controls and from the two homozygous patients by the Maxam–Gilbert method, using an end-labeled primer in the PCR, and by the dideoxynucleotide chain-termination method after subcloning. The two methods yielded identical results. Surprisingly, the DNA of both of the homozygous mutants contained an insertion/deletion that replaced 14 bp in the controls with 12 bp of unrelated sequence in the mutants (Fig. 3 *Upper*). Furthermore, when we sequenced PCR-amplified cDNAs prepared from total RNA isolated from cultured skin fibroblasts, the cDNA sequence showed exactly the same difference between the controls and both of the homozygous mutants as did the genomic sequences. Genomic DNA sequences obtained from the patients heterozygous for the 2.7-kbp band (patients 467, 519, 534, 543, 1747, and 1752) showed the same insertion/deletion in one copy of the *PCCB* gene.

Codon Deletion. Additional DNA sequence analysis in patients heterozygous for the insertion/deletion allele revealed a second type of mutation in the same region of the

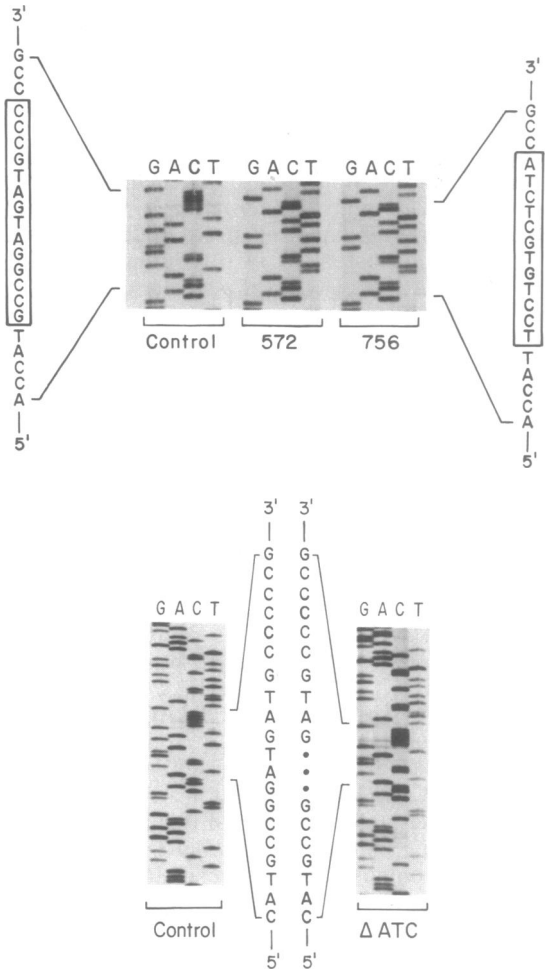

FIG. 3. Comparison of DNA sequences of PCR-amplified genomic DNAs from control and patients. Genomic DNAs were amplified by PCR, subcloned, and sequenced by the dideoxynucleotide chain-termination method (antisense strands shown). (*Upper*) Sequences derived from a control subject and from two patients (572 and 756) homozygous for the insertion/deletion. The boxed sequences show the differences: 14 nucleotides from control, 12 nucleotides from mutants (572 and 756). (*Lower*) Sequences derived from a control subject and from a patient (467) doubly heterozygous for the 3-bp in-frame deletion (ΔATC) and the insertion/deletion. Only the sequence of a subclone bearing the 3-bp deletion is shown. The three dots indicate the position of the deletion.

coding sequence. In this case, a 3-bp in-frame deletion of ATC was observed (Fig. 3 *Lower*), affecting one of two identical codons preceding the *Msp* I site. This eliminates one of two consecutive isoleucine residues in the predicted protein sequence (see Fig. 5). This deletion has, thus far, been found in only two patients (467 and 1752) from the *pccC* complementation group.

Blot Analysis of PCR-Amplified Products by ASO Hybridization. A wild-type oligonucleotide (16-mer) flanking the *Msp* I site hybridized with PCR products amplified from genomic DNA from controls and mutants heterozygous for the 2.7-kbp band but did not hybridize with those from homozygous mutants (Fig. 4). On the other hand, an oligonucleotide containing the inserted sequence showed clear hybridization with homozygous and heterozygous mutants and no hybridization with controls.

DISCUSSION

The most frequently seen mutations at CpG dinucleotides, as in an *Msp* I site (CCGG), are C → T or G → A transitions (20). Point mutations at CpG dinucleotides have been described in

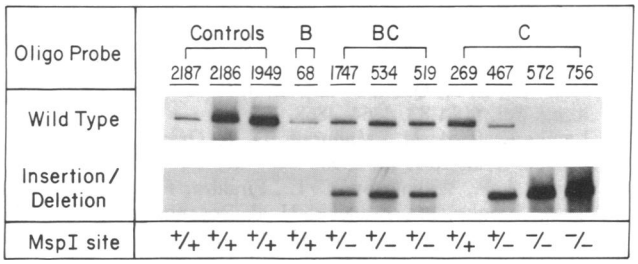

FIG. 4. ASO hybridization to Southern blots of PCR-amplified genomic DNAs of controls, one *pccB* patient, three *pccBC* patients, and four *pccC* patients. PCR products were separated in a denaturing 6% polyacrylamide gel (8 M urea), transferred electrophoretically to a nylon membrane, and probed with ^{32}P-labeled ASOs. + = positive, − = negative for the *Msp* I site. The sequences of the two probes were as follows: wild-type, 5′-ACCATGCCGGATGATG-3′ (antisense); insertion/deletion, 5′-ATTCCTGTGCTCTACC-3′ (antisense).

many inherited diseases (e.g., refs. 21 and 22). In this study, however, we have not detected such a point mutation, but rather have uncovered an insertion/deletion in the *PCCB* gene that coincidently affects an *Msp* I site. This insertion/deletion mutation replaces 14 nucleotides in control DNA with 12 nucleotides unrelated to this region in the mutant allele (Fig. 5). Furthermore, this insertion/deletion is a common mutation in the *pccBC* complementation group; it appeared in *pccBC* and *pccC* patients with an allele frequency estimated as 8 out of 22 alleles in these two subgroups, or 8 out of 28 alleles in the βPCC-deficiency group as a whole. All of the patients studied were Caucasian, largely from the United States.

The two homozygous mutants, 572 and 756, are siblings; however, six other, unrelated patients (467, 519, 534, 543, 1747, and 1752) with β-subunit deficiency shared the same insertion/deletion in one of their mutant alleles (data not shown). Two of them (467 and 1752) were also heterozygous for a 3-bp in-frame deletion (ΔATC) adjacent to, but not involving, the *Msp* I site. Such a high frequency of mutations in the same region among apparently unrelated patients suggests either (*i*) that there is a propensity for the *PCCB* gene to undergo mutations of this sort at this position or (*ii*) that the mutations, particularly the insertion/deletion, in all of the patients have a common origin in preceding generations.

The frequent insertion/deletion noted in our patients putatively results in a frameshift and a new stop codon in the transcribed mRNA about 30 bp downstream from the *Msp* I site. The predicted β subunit is shorter than the normal subunit by ≈100 amino acid residues. Western blotting of fibroblast extracts of these patients suggests that a small amount of shorter than normal subunits may be present, although quantitation and size estimation are difficult (data not shown). Because we did not detect any further variation in the sequence of the insertion/deletion alleles from ≈380 bp 5′-wards to ≈590 bp 3′-wards in the genomic DNA, we suggest that the frameshift and consequent premature termination are responsible for the lack of activity and protein instability that result in PCC deficiency in these patients.

The effect at the protein level of the loss of a single isoleucine residue due to the 3-bp deletion is not known. It has not been detected in at least 36 other *PCCB* alleles thus far sequenced. Because other mutations in this region have deleterious effects on βPCC, we propose that this single

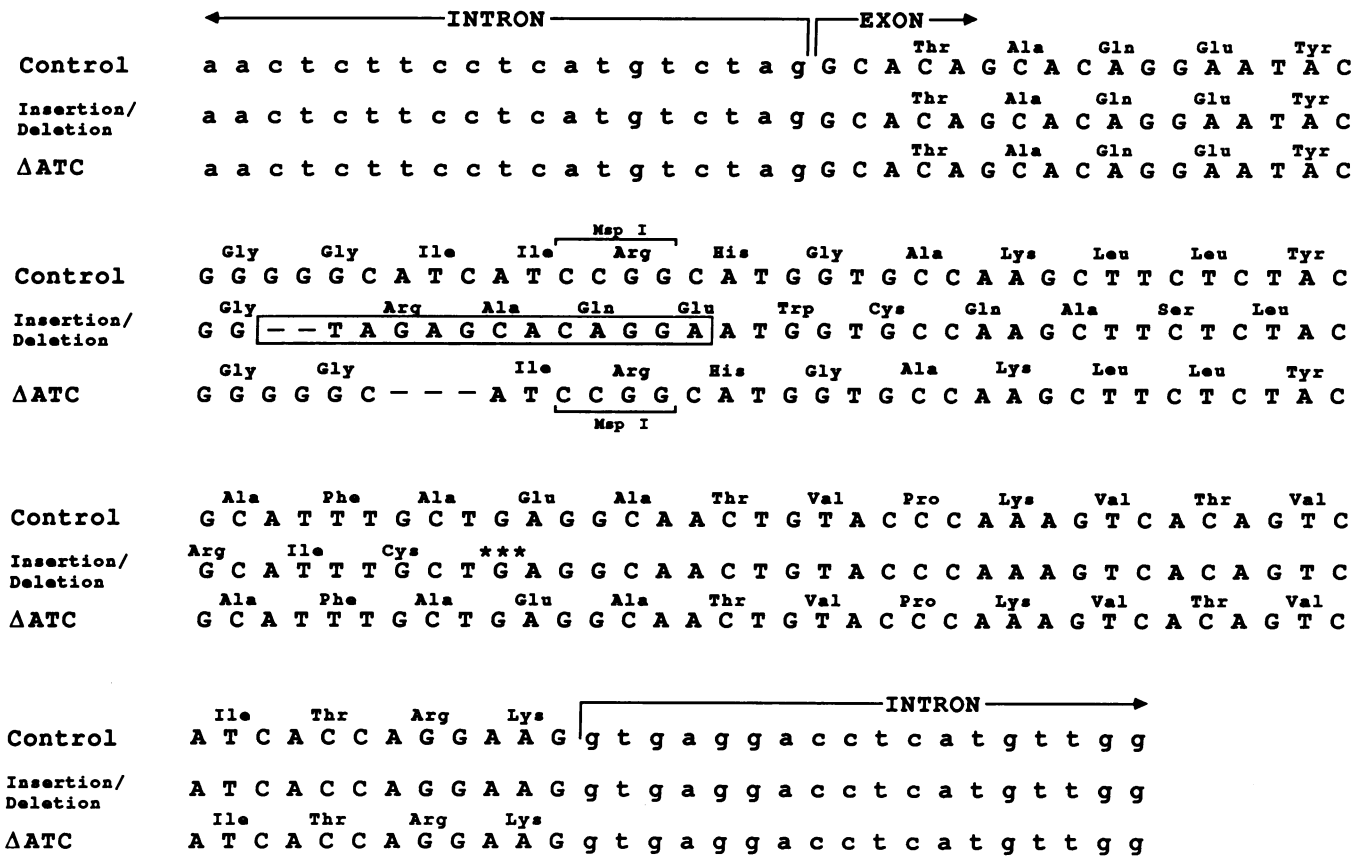

FIG. 5. DNA and predicted amino acid sequences of control and two mutations near the *Msp* I site. The *Msp* I site in the control sequence is shown. The 12 boxed nucleotides are the insertion that results in a net 2-bp deletion and a frameshift in the insertion/deletion allele. The three asterisks indicate the position of the new predicted stop codon in this allele. The three dashes in the ΔATC sequence indicate the deletion of one of the two adjacent isoleucine codons in this variant.

amino acid deletion is the second disease-causing mutation in the two compound heterozygotes.

The mechanism by which the insertion/deletion arose and the origin of the 12 inserted nucleotides remain undefined. As shown by the ASO hybridization (Fig. 4), an oligonucleotide containing the 12 inserted nucleotides reacts strongly with the PCR products from the two patients homozygous for the insertion/deletion, less well with heterozygotes, and not at all with controls. This indicates that the origin of the insertion is not in the immediate region of the mutation. Finally, our results show that common mutations and polymorphisms encountered at CpG dinucleotides, resulting in a loss of *Msp* I or *Taq* I sites, for example, are not always due to deamination of methylated cytosines.

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