Identification of a Second Mutation in the Protein-coding Sequence of the Z Type Alpha 1-Antitrypsin Gene*

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This study reports the entire nucleotide sequence of the protein coding region sequence of the alpha 1-antitrypsin (a1AT) Z gene, a common form of the a1AT gene associated with serum a1AT deficiency. In addition to Glu342 to Lys342 mutation in exon V which has been previously identified by peptide analysis, another point mutation (GTG to GCG in exon III) in the gene sequence predicts a second amino acid substitution (Val213 to Ala213) in the Z protein. This Val213 to Ala213 mutation was confirmed to be a general finding in Z type a1AT gene by evaluating genomic DNA from 40 Z haplotypes using synthetic oligonucleotide gene probes directed toward the mutated exon III sequences in the Z gene. Furthermore, the exon III Val213 to Ala213 mutation eliminates a BstEII restriction endonuclease site in the a1AT Z gene, allowing rapid identification of this Val213 to Ala213 substitution at the genomic DNA level. Surprisingly, when genomic DNA samples from individuals thought to be homozygous for the M1 gene (the most common a1AT normal haplotype) were evaluated with BstEII, 23% of the M1 haplotypes were BstEII site negative, thus identifying a new form of M1 (i.e. M1(Ala213)), likely identical to M1 but with an isoelectric focusing “silent” amino acid substitution (Val213 to Ala213). Although the relative importance of the newly identified exon III Val213 to Ala213 mutation to the pathogenesis of the abnormalities associated with the Z gene is not known, it is likely that M1(Ala213) gene represents a common “normal” polymorphism of the a1AT gene that served as an evolutionary intermediate between the M1(Val213) and Z genes.

Alpha 1-antitrypsin (a1AT) is an antiprotease that functions primarily as an inhibitor of neutrophil elastase, an omnivorous protease capable of destroying most forms of connective tissue (1). Coded for by a 10.2 kb at the chromosomal segment of 14q31-32 (2), the a1AT gene is comprised of five exons and four introns (3). The a1AT gene is expressed in liver hepatocytes and in mononuclear phagocytes as a 1.75-kb mRNA that is translated and secreted into the rough endoplasmic reticulum as a 418-amino acid precursor protein containing a 24-residue signal peptide (3-6). In the rough endoplasmic reticulum, N-linked carbohydrates are added to each of 3 asparaginyl residues, the protein is translocated to the Golgi where the high mannose carbohydrates are trimmed, and the glycosylated a1AT is secreted as a mature protein of 394 amino acids (5-8). The mature protein circulates in the plasma with a half-life of approximately 5 days (9, 10), and it diffuses into all tissues where it functions to inhibit neutrophil elastase.

The a1AT gene is highly pleomorphic with more than 30 known haplotypes of a1AT identified by isoelectric focusing (IEF) of serum (11–13). The two parental haplotypes are codominantly expressed. The most common haplotypes are of the M-family, including M1 (haplotype frequency in the United States Caucasian population 68-76%), M2 (14-20%), and M3 (10-12%) (11, 14, 15). Inheritance of any homozygous or heterozygous combinations of the M-family proteins is associated with “normal” levels of a1AT (150-350 mg/dl) (11), and they function similarly as excellent inhibitors of neutrophil elastase (16), with an association rate constant in the order of 10^7 M^-1 s^-1 (17).

In contrast to the M-family haplotypes, the Z haplotype is associated with low plasma levels of a1AT, i.e. “a1AT deficieny” (11, 13). Typically, individuals homozygous for the Z protein have a1AT levels 10-15% of normal (11). The Z gene represents 1-2% of all a1AT haplotypes of individuals of European descent (14, 15). Importantly, the ZZ homozygous state is associated in children with neonatal hepatitis, cholestasis, and cirrhosis, and in adults, with emphysema developing by ages 30-40 (11, 18-20). The emphysema is thought to develop because there is insufficient a1AT available to protect the fragile alveolar structures from their burden of neutrophil elastase; as a result there is slow, progressive destruction of the alveolar walls from the uninhibited elastase (19).

Since the observation in 1976 that a trypsin digest of the Z protein contained a single amino acid substitution (M1 Glu424 to Z Lys424), it has been assumed that this substitution is responsible for the deficiency and associated clinical manifestations of the ZZ homozygous state. As part of a general evaluation of a1AT gene structure associated with a1AT deficiency, we have cloned and sequenced the Z a1AT gene. To our surprise, we found that in addition to the “classic” exon V Glu424 to Lys424 substitution, the Z gene contains a second amino acid mutation (exon III, Val213 to Ala213). Further...
thence, through the evaluation of genomic DNA of what were thought to be α1AT M1 homoygotes, we have identified a new common form of α1AT type M1 which shares the Val\(^{213}\) to Ala\(^{213}\) mutation with the Z gene but has the same Glu\(^{42}\) sequence as the common M1 gene.

**EXPERIMENTAL PROCEDURES**

**Sources of Genomic DNA**—Genomic DNA was isolated from white blood cells of individuals with various α1AT phenotypes by the method of Jeffrey and Flavell (21). The α1AT phenotypes were identified by separation of serum IEF, serum α1AT levels, and family studies (13, 22). The α1AT serum levels were measured by radial immunodiffusion using the commercial standard (Behring Diagnostics). In addition to the α1AT heterozygote M3Z used for the cloning of the Z gene, genomic DNA was evaluated from 26 individuals with the serum phenotype M1M1 and 20 individuals with the serum phenotype ZZ. As controls, DNA was evaluated from individuals with haplotype M2 (n = 18), M3 (n = 6), and S (n = 7).

**Cloning and Sequencing of the Protein-coding Sequence of the Z Type α1AT Gene**—Using complete EcoRI digestion, a 10-kb EcoRI fragment of genomic DNA from an individual with the α1AT phenotype M3Z encompassing the entire protein-coding regions (exons II–V) of the α1AT gene (3) was cloned into AgtWES as described previously (20). The Z and M3 clones were identified by hybridization with 18-mer oligonucleotide gene-specific probes specific for the DNA sequences complementary to the amino acid sequences centered about the Glu\(^{42}\) (Z gene) and Glu\(^{42}\) (M3 gene) (23, 24). The 10-kb Z clone was digested into three fragments with PstI (1.6 kb containing exons II, 2.4 kb containing exons III and IV, and 1.1 kb containing exon V) and subcloned into pUC13. The double-stranded plasmid DNA with the insert was directly sequenced by the dideoxynucleotide chain termination method using bidirectional primers (25); 12,15-mer oligonucleotides were used to cover the sense sequence and 13,15-mer oligonucleotides to evaluate the antisense sequence of exons II–V and neighboring intron regions (9).

**Evaluation of α1AT Genes for Restriction Fragment Length Polymorphisms**—After sequencing of the Z α1AT gene demonstrated a mutation at residue 213 (see “Results”), it became apparent that the Z protein had a different charge than what was then called the M protein (29). In 1976, a tryptic peptide of the Z protein was found to differ from the corresponding peptide of the M protein by a loss of a glutamic acid residue and an addition of a lysine (30). Subsequent sequencing of an 8-amino acid segment of this region of the Z and M proteins confirmed the Glu to Lys substitution (31). Finally, when the entire M1 protein was sequenced by Carrell et al. (32), the partial human cDNA and the entire baboon cDNA by Kurachi et al. (33), and the entire M1 cDNA by Long et al. (3), it became apparent that the involved glutamic acid was situated at residue 342. The universality of the Glu\(^{42}\) to Lys\(^{42}\) (GAG to AAG in the genome) difference in all Z genes was confirmed at the genomic level by Kidd et al. (24) and by Nukiwa et al. (23) using 19-mer oligonucleotide probes centered about the Glu\(^{42}\) to Lys\(^{42}\) substitution.

With this as a background, despite the fact that more than 60% of the Z protein gene had not been sequenced (12, 31, 34), it has been generally assumed that the Glu\(^{42}\) to Lys\(^{42}\) substitution was the only difference in the primary structure between the Z and M1 proteins (32). However, when we sequenced the entire protein-coding exon region of the Z α1AT gene, we found that in addition to the classic exon V Glu\(^{42}\) to Lys\(^{42}\) substitution, the Z gene contained a second substitution (exon III, Val\(^{213}\) to Ala\(^{213}\); Fig. 1). In addition, the Z gene contains a silent base change in exon II (AAG to AAU) that codes for Lys\(^{342}\) in both the M1 and Z proteins.

Inheritance of the Z α1AT gene has several consequences: 1) the Z protein aggregates in the rough endoplasmic reticulum of the α1AT secreting cells (35, 36); 2) there is a reduced rate of secretion of the molecule by these cells (6, 37–39); 3) the plasma levels of α1AT are markedly reduced (28); and 4) the Z protein does not function as well as an inhibitor of neutrophil elastase (40). All available evidence suggests that in the ZZ homozygous state that the Z gene is transcribed in a normal fashion, that α1AT synthesizing cells have normal levels of α1AT mRNA, and that the Z type mRNA can be translated in a normal fashion (6, 37–39). However, studies with liver and mononuclear phagocytes of such individuals have shown that these cells secrete less α1AT than those of normals (6, 39). Consistent with this fact, light microscopic evaluation of biopsies of liver of ZZ individuals demonstrates intracellular accumulation of α1AT, and transmission electron microscopic evaluation of these specimens has shown that the α1AT accumulates in the rough endoplasmic reticulum (36). Furthermore, evaluation of the intracellular form of α1AT recovered from such livers demonstrated that it contains “high mannose” carbohydrate side chains (41). Together, this evidence has led to the concept that as the Z protein is produced, N-linked carbohydrates are normally added. However, liver accumulation and the plasma deficiency associated with the homozygous Z state result from a decreased rate of folding of the high mannose form of α1AT in the rough endoplasmic reticulum, allowing hydrophobic residues in adjacent molecules to interact, leading to aggregation. Interestingly, those Z type α1AT molecules that are translocated to the Golgi under normal trimming of the carbohydrate side chains, and such molecules are normally secreted (41) and have a normal circulating half-life (9). However, a recent study by Ogushi et al. (40) has demonstrated that the Z type molecule has a significantly reduced association rate constant for neutrophil elastase. In this context, in addition to the fact that the ZZ homozygous state is associated with a marked reduction in α1AT levels, on the average, the Z type molecule takes longer than does the M type molecule to inhibit an equivalent amount of neutrophil elastase.

The relative importance of the newly identified Val\(^{213}\) to
Ala mutation compared to the classic Glu212 to Lys212 mutation to each of these abnormalities associated with the Z protein is not known. The normal α1AT protein has been crystallized and its three-dimensional structure determined, but the three-dimensional structure of the Z protein has not been evaluated (42). In the M protein structure, the Glu242 residue is located in sheet A strand 5 and Val213 residue at the turn of segment 202–223 which forms a strongly twisted, double-stranded antiparallel ladder. It has been hypothesized that the Glu242 to Lys242 substitution results in a loss of a critical salt bridge (Glu242 to Lys246) which has an effect on the rate folding of the inhibitor, perhaps explaining the reduction in the rate of three-dimensional folding of the Z protein in the rough endoplasmic reticulum. The Val213 does not appear to participate in any critical salt bridges, nor does it appear in the three-dimensional structure near the active site at Met238. It is, however, reasonably close (in the tertiary structure) to Asn247 and hence to a carbohydrate attachment site (42). Whether this has any consequence to the intracellular handling of the molecule, or whether the Val213 to Ala213 substitution (or the Glu242 to Lys242 substitution) has any affect on the association rate constant of the interaction with neutrophil elastase, is unknown.

Despite the fact that the importance of the Val213 to Ala213 substitution is not known, the knowledge of its presence has led us to the identification of a previously unrecognized, but common polymorphic form of the normal M1 gene. Evaluation of the normal M1 gene sequence in the Val213 region revealed that the endonuclease BstEII normally cuts in the sequences in exon III coding for the amino acids Glu213–Val213–Thr214. Theoretically, however, with the substitution GTG to GCC (Val213 to Ala213) in the Z protein, this BstEII restriction site would be lost. Evaluation of genomic DNA from individuals homozygous for the M1 gene and those homozygous for the Z gene demonstrated this to be the case. In this context, if the Z gene is cut with PstI and BstEII, there is no BstEII site in exon III, and thus a single 0.95-kb fragment is generated that can be detected with an exon III probe (Fig. 2, lane 1). In contrast, in the M1 gene, the presence of the exon III BstEII site leads to the generation of a 0.72-kb fragment (Fig. 2, lane 2; a 0.23-kb fragment is also generated, but it does not appear on the autoradiogram because it does not bind efficiently to the filter). We initially thought this loss of a restriction site associated with the Z gene would be useful as a method to uniquely identify the Z gene from the common M-family haplotypes. However, in evaluating this hypothesis, we soon realized that a significant proportion of genomic DNA samples that had been identified as being M1M1 homozygotes by conventional criteria (13) could be further subgrouped depending on whether they contained or did not contain the BstEII restriction site. In this context, some M1M1 samples contained the BstEII site (Fig. 2, lane 2), while others were homozygous for the absence of this site (Fig. 2, lane 3), and still others were heterozygous for this site (Fig. 2, lane 4). However, when M2, M3, and S haplotypes were evaluated, all were BstEII positive (i.e. all have the same sequence in the 213 region as the classic M1 gene; data not shown). Thus, it became apparent that α1AT haplotypes thought to be M1, can actually be M1(Val213) or M1(Ala213).2

Comparison of the IEF patterns of serum of individuals homozygous for M1(Val213) and M1(Ala213) demonstrated they were identical (data not shown), as might be expected by a

2 An unpublished sequence of an α1AT cDNA (S. L. C. Woo, and E. W. Davie) referred to by Carrell et al. (32) and also by Rosenberg et al. (45) showed an Ala at amino acid 213; presumably this cDNA represents M1(Ala213).

[Fig. 2. Identification of a restriction fragment length polymorphism in the Z α1AT gene resulting from the exon III Val213 mutation and the identification of a form of the M1 gene (M1(Ala213)) with this same polymorphism. Shown at the top is a schematic of the α1AT gene with its five exons (solid boxes, I–V) and four introns. The exon III Val213 mutation is indicated by ▽. Based on the sequence of the M1 and Z genes (Fig. 1), indicated are the expected restriction sites for the enzymes PstI (P) and BstEII (B) and the predicted fragments that should be generated from M1 (0.72, 0.23 kb) and Z (0.95 kb) genomic DNA, respectively. Shown at the left is the sequence of the M1 and Z genes around the 213 region, indicating the expected cutting site of BstEII in the M1 gene and the expected loss of this site in the Z gene. After the DNA was cut with the two enzymes, the resulting fragments were analyzed by Southern transfer (44) and hybridized with a 32P-labeled exon III probe (see "Experimental Procedures"). Lane 1, genomic DNA from an individual with the α1AT phenotype ZZ showing only the expected 0.95-kb fragment. Lane 2, genomic DNA from an individual with the phenotype M1(Ala213) showing only the expected 0.72-kb fragment. The expected 0.23-kb band (>) does not appear on the autoradiogram because this small fragment does not bind to a nitrocellulose filter efficiently. Lane 3, genomic DNA from an individual with the α1AT phenotype M1(Val213)M1(Ala213). This individual has the protein phenotype M1M1, but with the BstEII 0.95-kb fragment instead of the expected 0.72-kb fragment; this identifies a newly recognized polymorphic form of M1 that does not have the BstEII restriction site in exon III (referred to as M1(Ala213)). Lane 4, genomic DNA from an individual heterozygous for M1(Val213) and M1(Ala213).]
**Alpha 1-Antitrypsin Z Gene**

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**Fig. 3.** Detection of the exon III and exon V sequence differences of the Z, M1(Valz13), and M1(Ala213) α1AT genes using oligonucleotide probes. At the top of the figure is a schematic of the α1AT gene showing the five exons (*solid boxes labeled I-V*), the putative promoter site 5' to exon I, the start codon in exon II, and the stop codon in exon V. With EcoRI (E) and BglII (B), the five exons are conveniently cut into five different size DNA fragments, each containing a single exon. Thus, after Southern transfer and hybridization with a 32P-labeled full length α1AT cDNA (45), an autoradiogram reveals fragments of 4.3, 2.0, 1.5, 1.3, and 0.9 kb correspon to exons II, I, IV, V, and III, respectively (23) (far left lane, labeled Probe, cDNA). The exon III Val213-Ala213 substitution (M1(Val213) to M1(Ala213) or Z) is caused by a base change of GTG to GCG. The 19-mer oligonucleotide gene probes used to detect this change are indicated as the "exon III Val213 probe" and "exon III Ala213 probe." From the BstEII data (Fig. 2), it would be expected that the exon III Val213 probe would hybridize to the M1(Val213) haplotype but not to the Z or M1(Ala213) haplotype while the exon III Ala213 probe would hybridize in the reverse order. The exon V Glu342 to Lys mutation (M1(Val213) or M1(Ala213) to Z) is caused by a base change of GAG to AAG. The 19-mer oligonucleotide gene probes used to detect this change are indicated as the "exon V Glu342 probe" and the "exon V Lys342 probe." From prior studies (23, 24), it is known that the exon V Glu342 probe hybridizes to all M1 genes tested (presumably including M1(Ala213) as well) but not to the Z gene, while the exon V Lys342 probe hybridizes to all Z genes but not M1 (and presumably M1(Ala213)). All oligonucleotide probes were labeled with incorporation of [32P]dNTPs by E. coli DNA polymerase (Klenow fragment; Pharmacia) using reverse complementary templates and a small 8-mer primer (27). Bases in the probes that are labeled are indicated by *. Genomic DNA from various sources were cut with the endonucleases BglII and EcoRI, 5 µg were electrophoresed, transferred to nitrocellulose, hybridized with a labeled probe, washed and autoradiographed. Shown are the evaluations of genomic DNA from individuals with various α1AT phenotypes. (A) ZZ (isoelectric focusing of serum pattern ZZ, genomic DNA BstEII pattern -/-); note hybridization with the exon III Ala213 probe and exon V Lys342 probe, but not with the exon III Val213 probe or exon V Glu342 probe. (B) M1(Val213)M1(Val213) (isoelectric focusing pattern M1M1, genomic DNA BstEII pattern +/-); note hybridization with the exon III Ala213 probe and exon V Lys342 probe, but not with the exon III Val213 probe or exon V Glu342 probe. (C) M1(Ala213)M1(Ala213) (isoelectric focusing pattern M1M1, genomic DNA BstEII pattern -/-); note hybridization with the exon III Ala213 probe and exon V Glu342 probe, but not with the exon III Val213 probe or the exon V Lys342 probe.
The exon III VaP3 probe was found to be more frequent than the M3 probe (14, 15). Like its unknown importance to the Z gene or protein, the functional importance of the Ala213 to Ala213 mutation to the M1 gene or protein is unknown. However, our preliminary studies comparing the individuals homozygous for M1(VaP3)M1(VaP3) to those homozygous for M1(Ala213)M1(Ala213) have failed to reveal any marked differences in α1AT levels or function.

The fact that the Z gene differs from the M1(VaP3) gene by more than one mutation, and that some individuals thought to have the M1(VaP3) α1AT haplotype actually have the M1(Ala213) haplotype, leads to two interesting conclusions. First, since the Z sequence differs from the M1(VaP3) sequence at two sites (amino acids 213 and 342), the Z gene could not have evolved from the M1(VaP3) gene (or vice versa) directly by a single mutational event (Fig. 4). Second, the available evidence suggests that the M1(Ala213) gene was an evolutionary intermediate between the M1(VaP3) and Z genes. In this regard, the M1(Ala213) gene sequence is identical to the baboon α1AT sequence (53) at the codons Lys219 (AAG), Ala233 (GCC), and Glu342 (GAG). In contrast, the M1(VaP3) sequence differs from the baboon at one codon (M1(VaP3) (GTG), baboon Ala213 (GCG)) and the Z gene differs from the baboon at two codons (Z Lys219 (AAA), Z Lys342 (AAG), baboon Glu213 (GAG)) (Fig. 4).

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### Table 1

Comparison of the alpha 1-antitrypsin BstEII patterns and oligonucleotide evaluation of alpha 1-antitrypsin sequences centered at amino acid residues 213 and 342

| Serum isoelectric focusing pattern | BstEII pattern | Phenotype | n | Exon III VaP3 probe | Exon III Ala213 probe | Exon V Glu342 probe | Exon V Lys444 probe |
|-----------------------------------|----------------|-----------|---|---------------------|----------------------|----------------------|---------------------|
| M1M1 (+/+)                        | M1(VaP3)M1(VaP3) | 16        | 16 | 16                  | 0                    | 16                   | 0                   |
| M1M1 (+/-)                        | M1(VaP3)M1(Ala213) | 8         | 8  | 8                   | 8                    | 8                    | 8                   |
| M1M1 (−/−)                        | M1(Ala213)M1(Ala213) | 2         | 0  | 2                   | 2                    | 2                    | 2                   |
| ZZ                                 | ZZ              | 20        | 20 | 0                   | 0                    | 20                   | 20                  |

* Oligonucleotide evaluation was carried out as described in the legend to Fig. 3. Note the exact correlation among the BstEII patterns and the oligonucleotide patterns.

** The BstEII pattern of genomic DNA was determined as described in the legend to Fig. 2. +/+ = 0.72-kb fragment present, 0.95-kb fragment absent; +/− = both 0.72- and 0.95-kb fragments present; −/− = 0.95-kb fragment present, 0.72-kb fragment absent.

The alpha 1-antitrypsin phenotype was determined by a combination of IEF, serum levels, family studies, and genomic DNA BstEII patterns. M1(VaP3)M1(VaP3) = IEF pattern M1M1, normal serum levels, BstEII +/-; M1(VaP3)M1(Ala213) = IEF pattern M1M1, normal serum levels, BstEII +/-; ZZ = IEF pattern ZZ, serum levels <50 mg/dl, BstEII +/-.
FIG. 1. Sequence of the protein coding regions of the α1ZT Z gene. Shown is the sequence of the protein coding exons (II-V) of the Z α1ZT gene; for clarity it is shown in a continuous form from one exon to the next, with dotted vertical lines separating the exons. The translated amino acid sequence for the Z gene is immediately below the nucleotide sequence. The numbering starts from N-terminal glutamic acid residue of the mature α1ZT protein; amino acids within the signal peptide are identified with + and the carbohydrate attachment sites are indicated in vertical lines.

APPENDIX

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